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(54) Title: CAPON: A PROTEIN ASSOCIATED WITH NEURONAL NITRIC OXIDE SYNTHASE

## (57) Abstract

Nitric oxide (NO) produced by neuronal nitric oxide synthase (nNOS) is important for N-methyl-D-aspartate (NMDA) receptor-dependent neurotransmitter release, neurotoxicity, and cyclic-GMP elevations. The coupling of NMDA receptor-mediated calcium influx and nNOS activation is postulated to be due to a physical coupling of the receptor and the enzyme by an intermediary adaptor protein PSD95, through a unique PDZ-PDZ domain interaction between PSD95 and nNOS. Here we report the identification of a novel nNOS associated protein, CAPON, which is highly enriched in brain and has numerous colocalizations with nNOS. CAPON interacts with the nNOS PDZ domain through its C-terminus. CAPON competes with PSD95 for interaction with nNOS, and overexpression of CAPON results in a loss of PSD95/nNOS complexes in transfected cells. CAPON influences nNOS by regulating its ability to associate with PSD95/NMDA receptor complexes.

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## CAPON: A PROTEIN ASSOCIATED WITH NEURONAL NITRIC OXIDE SYNTHASE

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### TECHNICAL AREA OF THE INVENTION

The invention relates to the area of neurotransmitter regulation. More particularly, the invention relates to the regulation of neuronal nitric oxide synthase.

### BACKGROUND OF THE INVENTION

Studies of neuronally-derived nitric oxide (NO) have revealed many roles for this gaseous messenger molecule (Moncada, 1994; Yun et al., 1996). In the peripheral nervous system, NO mediates nonadrenergic, noncholinergic neurotransmission, serving as an effector of autonomic neurons on smooth muscle. NO has been implicated in several forms of neuronal plasticity, such as LTP (for a review see Huang, 1997). Studies in mice with a targeted genomic deletion of the NO biosynthetic enzyme, neuronal NO synthase (nNOS), have shown that NO mediates a substantial portion of the neurotoxicity associated with stroke (Huang et al., 1994). In the brain, NO and citrulline are produced from arginine predominantly by a neuronal isoform of NOS (nNOS) (Huang et al., 1993), although endothelial NOS (eNOS) may also occur in neurons (Dinerman et al., 1994; O'Dell et al., 1994). Most neurotransmitters are

5 stored in synaptic vesicles and neurotransmitter effects are elicited following the exocytosis of transmitter into the synaptic space. For an evanescent transmitter such as NO there is no storage pool and newly synthesized NO is used as it is made. NO synthesis is triggered by the influx of calcium, which, when complexed with calmodulin, activates the biosynthetic activity of NOS (Bredt and Snyder, 1990).

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Because NO lacks vesicular storage and depends on new synthesis for its release, nNOS must be associated with the plasma membrane. Subcellular fractionation indicates that roughly half of brain nNOS is soluble and half particulate (Bredt, 1996; Hecker et al., 1994). Recently, Bredt and associates showed that nNOS is targeted to membranes by binding to syntrophin, PSD95/SAP90, or PSD93 (Brennan et al., 1996; Brennan et al., 1996). These proteins are enriched in synaptic densities and interact with nNOS through PDZ domains, consensus sequences of about 100 amino acids that are found in proteins which tend to be associated with cell-cell junctions (Ponting and Phillips, 1995). The nNOS/PSD95 interaction involves a portion of nNOS which includes its sole PDZ domain and the second PDZ domain of PSD95. PSD95 was first isolated from postsynaptic densities (Cho et al., 1992) but also occurs in presynaptic nerve terminals (Kistner et al., 1993) and clusters neurotransmitter receptors and ion channels at synaptic sites (Kornau et al., 1997). For instance, the NMDA receptor and several potassium channels are associated with PSD95 at synapses (Kornau et al., 1995). The linking of NMDA receptors to nNOS by PSD95 may explain why calcium influx following NMDA receptor activation leads to a tightly coupled nNOS activation (Brennan et al., 1996). Indeed, the effects of NO appear to be intimately tied to the NMDA receptor. For example, NMDA receptor-mediated neurotoxicity (Dawson and Dawson, 1996), neurotransmitter release (Schuman and Madison, 1994), and cGMP elevations (Bredt and Snyder, 1989; Garthwaite et al., 1989) each require nNOS and are blocked by nNOS-specific inhibitors. Moreover, NO can directly modulate NMDA receptors (Lipton and Stamler, 1994).

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There is a continuing need in the art of neurotransmitter regulation for methods of affecting the activity of neuronal NOS, so that one can manipulate NO levels when required for therapeutic effect in such disorders.

**SUMMARY OF THE INVENTION**

It is an object of the invention to provide an isolated mammalian Capon (Carboxy-terminal PDZ ligand of nNOS) protein.

5 It is another object of the invention to provide a fusion protein comprising at least eight contiguous amino acids selected from the Capon amino acid sequence shown in SEQ ID NO:2.

It is yet another object of the invention to provide an isolated polypeptide consisting of at least eight contiguous amino acids of Capon as shown in SEQ ID NO:2 and capable of binding an nNOS PDZ domain.

10 It is still another object of the invention to provide a preparation of antibodies which specifically bind to a Capon protein as shown in SEQ ID NO:2 or 4.

It is even another object of the invention to provide a subgenomic polynucleotide which encodes a Capon protein as shown in SEQ ID NO:2 or 4.

15 It is yet another object of the invention to provide a recombinant DNA construct for expressing Capon antisense nucleic acids.

It is still another object of the invention to provide a method of inhibiting a mammalian neuronal nitric oxide synthase (nNOS).

It is even another object of the invention to provide methods of screening test compounds for the ability to decrease or augment the activity of nNOS.

20 These and other objects of the invention are provided by one or more of the embodiments described below. One embodiment of the invention provides an isolated mammalian Capon protein which has the sequence shown in SEQ ID NO:2 or 4, and naturally occurring biologically active variants thereof.

25 Another embodiment of the invention provides a mammalian Capon fusion protein which comprises two protein segments fused to each other by means of a peptide bond, wherein one of the protein segments consists of at least eight contiguous amino acids selected from the amino acid sequence shown in SEQ ID NO:2 or 4.

30 Yet another embodiment of the invention provides an isolated polypeptide which consists of at least eight contiguous amino acids of Capon as shown in SEQ ID NO:2 or 4, wherein the polypeptide binds to an nNOS PDZ domain.

Still another embodiment of the invention provides a preparation of antibodies which specifically bind to a mammalian Capon protein as shown in SEQ ID NO:2 or

4.

Even another embodiment of the invention provides a subgenomic 5 polynucleotide which encodes a Capon protein as shown in SEQ ID NO:2 or 4.

Yet another embodiment of the invention provides a recombinant DNA construct for expressing Capon antisense nucleic acids, comprising a promoter and a coding sequence for Capon consisting of at least 12 contiguous base pairs selected from SEQ ID NO:1 or 3, wherein the coding sequence is in an inverted orientation 10 with respect to the promoter, such that upon transcription from the promoter an RNA is produced that is complementary to native mRNA encoding Capon.

Still another embodiment of the invention provides a method of decreasing a mammalian nNOS activity, comprising the step of contacting a nNOS with a Capon protein having an amino acid sequence as shown in SEQ ID NO:2 or 4.

15 Even another embodiment of the invention provides a method of screening test compounds for the ability to decrease or augment nNOS activity. The method comprises the steps of: (a) contacting a test compound with a mixture of a mammalian Capon protein and a polypeptide comprising an nNOS PDZ domain; and (b) measuring the amount of Capon or the polypeptide that is bound or unbound in the presence of 20 the test compound, a test compound that decreases the amount of bound Capon or the polypeptide being a potential drug for increasing nNOS activity, and a test compound that increases the amount of the polypeptide or Capon that are bound being a potential drug for decreasing nNOS activity.

Yet another embodiment of the invention provides a method of screening test 25 compounds for the ability to decrease or augment nNOS activity comprising the steps of: (a) contacting a cell with a test compound, wherein the cell comprises: i) a first fusion protein comprising (1) a DNA binding domain or a transcriptional activation domain, and (2) all or a portion of a mammalian Capon protein, wherein the portion consists of a contiguous sequence of amino acids selected from the amino acid sequence shown in SEQ ID NO:2 or 4, wherein the portion is capable of binding to nNOS; ii) a second fusion protein comprising (1) a transcriptional activation domain

or a DNA binding domain and (2) all or a portion of nNOS, wherein the portion comprises a PDZ domain; or a naturally occurring biologically active variant thereof, wherein the interaction of the portion of the Capon protein with the portion of nNOS reconstitutes a sequence-specific transcriptional activating factor, wherein when the 5 first fusion protein comprises a DNA binding domain the second fusion protein comprises a transcriptional activation domain and when the first fusion protein comprises a transcriptional activation domain the second fusion protein comprises a DNA binding domain; and iii) a reporter gene comprising a DNA sequence to which the DNA binding domain of the first fusion protein specifically binds; and (b) 10 measuring the expression of the reporter gene, a test compound that increases the expression of the reporter gene being a potential drug for decreasing nNOS activity, and a test compound that decreases the expression of the reporter gene being a potential drug for augmenting nNOS activity.

Even another embodiment of the invention provides a method of screening test 15 compounds for the ability to decrease or augment nNOS activity comprising the steps of: (a) contacting a cell with a test compound, wherein the cell comprises: (i) a first expression vector comprising a subgenomic polynucleotide encoding at least the PDZ domain of nNOS or a naturally occurring biologically active variant thereof; (ii) a second expression vector comprising a subgenomic polynucleotide encoding at least 20 the portion of Capon or a naturally occurring biologically active variant thereof, wherein the portion of Capon is capable of binding to nNOS; and (b) measuring the amount of cGMP, NO, or citrulline in the cell, a test compound that increases the amount of cGMP, NO, or citrulline being a potential drug for augmenting nNOS activity, and a test compound that decreases the amount of cGMP 25 being a potential drug for decreasing nNOS activity.

According to still another embodiment a method is provided for diagnosing a neurological disease or a propensity for a neurological disease, comprising: determining number of glutamine repeats present in a Capon protein of a patient wherein a number greater than 6 indicates a neurologic disease or a propensity 30 therefor.

According to still another embodiment a method is provided for diagnosing a neurological disease or a propensity for a neurological disease, comprising: determining number of CAG repeats in a *Capon* gene of a patient, wherein a number greater than 6 indicates a neurologic disease or a propensity therefor.

5 Another aspect of the invention is a cell comprising one or more recombinant nucleic acid molecules. The cell comprises: (i) a first expression vector comprising a subgenomic polynucleotide encoding at least the PDZ domain of nNOS or a naturally occurring biologically active variant thereof; and (ii) a second expression vector comprising a subgenomic polynucleotide encoding at least a portion of *Capon* or a naturally occurring biologically active variant thereof, wherein the portion of *Capon* 10 is capable of binding to nNOS.

15 Another aspect of the invention is a cell comprising one or more recombinant nucleic acid molecules. The cell comprises: i) a nucleotide construct encoding a first fusion protein comprising (1) a DNA binding domain or a transcriptional activation domain, and (2) all or a portion of a mammalian *Capon* protein, wherein the portion consists of a contiguous sequence of amino acids selected from the amino acid sequence shown in SEQ ID NO:2 or 4, wherein the portion is capable of binding to nNOS; ii) a nucleotide construct encoding a second fusion protein comprising (1) a transcriptional activation domain or a DNA binding domain and (2) all or a portion of 20 nNOS, wherein the portion comprises a PDZ domain, or a naturally occurring biologically active variant thereof, wherein the interaction of the portion of the *Capon* protein with the portion of nNOS reconstitutes a sequence-specific transcriptional activating factor, wherein when the first fusion protein comprises a DNA binding domain the second fusion protein comprises a transcriptional activation domain, and when the first fusion protein comprises a transcriptional activation domain the second 25 fusion protein comprises a DNA binding domain; and iii) a reporter gene comprising a DNA sequence to which the DNA binding domain of the first fusion protein specifically binds, wherein upon reconstitution of the sequence specific transactivating factor, expression of the reporter gene is increased.

30 The present invention thus provides the art with the information that *Capon*, a heretofore unknown protein, regulates the activity of neuronal nitric oxide synthase.

Capon can be used, *inter alia*, in assays to screen for substances which have the ability to decrease or augment neuronal nitric oxide synthase activity.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

##### **Figure 1. Cloning of the CAPON cDNA and distribution of CAPON mRNA.**

5 (Figure 1 A) CAPON specifically interacts with nNOS in the yeast two-hybrid system. Yeast were transformed with the indicated Gal4 activation domain (AD) and Gal4 DNA-binding domain (BD) plasmids and grown on plates containing histidine. A typical filter lift is shown in which  $\beta$ -galactosidase activity was detected by the appearance of a blue precipitate. pAD-CAPON1, comprising the last 125 amino acids 10 of CAPON, activates lacZ transcription in the presence of pBD-nNOS (2-377) but not with plasmids the first three PDZ domains of PSD95 (amino acids 20-364) or the second PDZ domain of PSD93 (amino acids 116-421).

15 (Figure 1 B) Amino acid sequence of rat CAPON and alignment with a partial human sequence. The underlined sequence corresponds to the putative PTB domain. The bracketed sequence is encoded by a CAG repeat. A human expressed sequence tag (EST) (accession number R19867) obtained from a library derived from infant brain contains a 459 bp open reading frame with homology to the C-terminus of the CAPON cDNA ( $p = 5.1 \times 10^{-17}$ ). The conceptual translation of this clone reveals a protein with 20 92% amino acid identity with the rat protein.

20 (Figure 1 C) CAPON is enriched in neuronal structures. Northern (RNA) blot analysis reveals that several CAPON transcripts are present, and these transcripts are enriched in neuronal tissues.

##### **Figure 2. Interaction of CAPON and nNOS.**

25 (Figure 2 A) CAPON binds to nNOS, but not eNOS or iNOS. Bacterially expressed GST, GST-PIN, and GST-CAPON were bound to glutathione agarose and then incubated with lysates of HEK293 cells transfected with expression plasmids for the indicated isoforms of NOS. After extensive washing of the resins, bound NOS was detected with isoform specific antibodies. While nNOS binds to both GST-PIN and GST-CAPON, neither eNOS or iNOS bind to either protein. Input = 10% of starting 30 material applied to each resin.

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**(Figure 2 B)** A GST-NOS fusion protein specifically binds to rat brain CAPON. A fusion protein consisting of GST and amino acids 1-100 of nNOS was bound to glutathione agarose and then incubated with cerebellar supernatants. After extensive washing, CAPON is detected on the NOS resin but not on the control GST resin. A second ~48 kD band is also detected with this antibody, but fails to interact with nNOS. This band may represent a cross-reactive protein, an alternatively spliced isoform of CAPON, or a degradation product.

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**(Figure 2 C)** CAPON interacts with nNOS directly. HEK293 lysates transfected with a expression plasmid containing the nNOS cDNA or empty vector were resolved by electrophoresis, transferred to nitrocellulose, and probed with radiolabeled CAPON (see Methods). Purified nNOS is recognized with this probe, along with a comigrating band detected in NOS-transfected but not mock-transfected cells.

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**(Figure 2 D)** CAPON and nNOS complexes are detectable in cerebellar lysates (I). Cerebellar supernatants were prepared from wild-type (+/+) and nNOS knockout (-/-) mice and incubated with the 2', 5', ADP-sepharose, an nNOS-affinity resin. Only CAPON derived from supernatants of wild-type and not knockout animals was capable of binding the resin indicating that the presence of nNOS is required for CAPON to bind to the resin. CAPON levels were decreased in knockout animals, presumably due to decreased stability in the absence of the nNOS binding partner. A lower molecular weight band (arrowhead), frequently detected with the anti-CAPON antibody, bound weakly to the resin in an nNOS-specific manner as well. Input = 20% of lysate used for binding.

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**(Figure 2 E)** CAPON and nNOS complexes are detectable in cerebellar lysates (II). An antibody to CAPON (5 mg) specifically coprecipitates nNOS, while comparable amounts of antibody to the G-protein subunit b1, cyclin-dependent kinase 2, and preimmune serum fail to coprecipitate nNOS. Enrichment of nNOS in CAPON immunoprecipitates was specific as a control protein, protein kinase C- b I/II did not display similar enrichment. Input = 10% of lysate used for immunoprecipitation.

**Figure 3.** Immunohistochemical localization of nNOS and CAPON.

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**(Figure 3 A)** Comparison of nNOS-IR (immunoreactivity), CAPON-IR, and CAPON in situ hybridization patterns in sagittal sections of adult rat. Islands of Calleja (solid arrowhead); supraoptic nucleus (open arrowhead); AOB, accessory olfactory bulb; C, colliculi; Cb, cerebellum; Cx, cerebral cortex; OB, olfactory bulb. Immunohistochemical nonspecific labelling ("Block") was determined using a CAPON antibody pre-absorbed with the antigenic peptide. Nonspecific hybridization ("Sense") was detected using a sense probe.

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**(Figure 3 B)** Comparison of cellular localization of nNOS-IR and CAPON-IR in adult rat brain. CAPON-IR (*a*) and nNOS-IR (*b*) hypothalamic neurons, solid arrowheads indicate IR dendritic processes. CAPON-IR (*c*) and nNOS-IR (*d*) of the supraoptic nucleus. CAPON-IR (*e*) and nNOS-IR (*f*) cell bodies of the nucleus of the trapezoid body separated by unreactive fascicles of nerve fibers. Adjacent is the pontine nucleus (Pn) which exhibits both CAPON and nNOS-IR (*e, f*). CAPON-IR (*g*) and nNOS-IR (*h*) in the cerebellum. Molecular cell layer (Mol); granular cell layer (Gr). Micrographs *e, f*, (100x); *c, d, g, h*, (200x); *a, b* (400x).

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**Figure 4.** The PDZ domain of nNOS interacts with the C-terminus of CAPON.

**(Figure 4 A)** The PDZ domain of nNOS (amino acids 20-100) is sufficient for binding to CAPON. Truncations of nNOS were subcloned into the Gal4 BD vector and nNOS/CAPON interactions were detected by  $\beta$ -galactosidase assays.

**(Figure 4 B)** The C-terminal 13 amino acids of CAPON are sufficient for binding to nNOS. Various GST-CAPON fusion proteins were incubated with HEK293 lysates containing nNOS. A CAPON fusion protein comprising the last 100 amino acids binds nNOS, as do fusion proteins comprising the last 13 or 20 amino acids of CAPON. A CAPON fusion protein with the last 20 amino acids deleted no longer binds nNOS.

Neither of the control proteins, GST or GST-14-3-3, are able to bind nNOS.

**(Figure 4 C)** Amino acid substitutions in the C-terminus of CAPON prevent it from interacting with nNOS. His<sub>6</sub>-fusion proteins of the last 100 amino acids of CAPON were generated and incubated with GST-NOS (1-100) immobilized on glutathione agarose. While the unmutagenized sequence binds (last four amino acids EIAV), mutation of the terminal valine (EIAA) or the penultimate alanine (EIDV) prevents

binding. Serine or alanine mutations are tolerated at the n-2 position (ESAV and EAAV), but truncation of the C-terminal 13 amino acids blocks binding altogether.

**Figure 5. CAPON and PSD95 compete for binding to nNOS.**

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**(Figure 5 A)** His<sub>6</sub>-CAPON fusion proteins specifically block the nNOS/PSD95 interaction in vitro. The C-terminal 100 amino acids were fused to a His<sub>6</sub> tag and added to HEK293 lysates transfected with nNOS expression plasmids at the indicated fusion protein concentration. This mixture was added to GST-PSD95 (amino acids 20-364) or GST-PSD93 (116-421), the regions of these proteins previously shown to interact with nNOS (Brenman et al., 1996). The disruption of the nNOS/PSD95 interaction required the C-terminal 13 amino acids, as this fusion protein (DC20) fails to block the interaction even at 5 mM. Other control proteins such as His<sub>6</sub>-PIN or His<sub>6</sub>-FKBP do not disrupt the nNOS-PSD95 interaction.

**(Figure 5 B)** Quantification of CAPON inhibition of the nNOS/PSD95 interaction. HEK293 cells were transfected with nNOS expression plasmids and then metabolically labeled with [<sup>35</sup>S] methionine. Radiolabeled nNOS was purified and mixed with His<sub>6</sub>-CAPON as in (A), above. Bound nNOS was resolved by electrophoresis and counts were determined on a PhosphoImager.

**Figure 6. CAPON expression prevents the interaction of PSD95 and nNOS.**

HEK293 cells were transfected with various combinations of expression plasmids for HA-tagged nNOS (HA-NOS), myc-tagged PSD95, or CAPON. Following transfection, the lysates were immunoprecipitated with an anti-HA antibody and bound proteins were detected with the appropriate antibodies. Following cotransfection of HA-NOS and myc-PSD95, myc-PSD95 is detected in anti-HA immunoprecipitates. Cotransfection of a full-length CAPON expression plasmid substantially reduces the amount of myc-PSD95 in anti-HA precipitates. In the absence of HA-nNOS transfection, neither myc-PSD95 or CAPON is immunoprecipitated by anti-HA antibodies.

**Figure 7. Model of PSD95/nNOS regulation by CAPON.**

NMDA receptors are coupled to nNOS through a PSD95 multimer. These interactions are mediated by PDZ domains. In this complex, nNOS is situated close to NMDA receptor-modulated calcium influx (left). Binding of CAPON (right) results in a

reduction of NMDA receptor/PSD95/nNOS complexes, leading to decreased access to NMDA receptor-gated calcium influx and a catalytically inactive enzyme.

### **DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

We conducted a yeast two-hybrid screen in which we have identified a novel 5 protein which we designate CAPON (Carboxyl-terminal PDZ ligand of nNOS). CAPON is a cytoplasmic protein whose carboxyl terminus binds to the PDZ domain of nNOS. CAPON competes with PSD95 and PSD93 for binding to nNOS and thus may participate in the translocation and impede the activation of this enzyme.

It is a discovery of the present invention that the mammalian protein Capon 10 (Carboxy-terminal PDZ ligand of nNOS) physically interacts with and inhibits the activity of neuronal nitric oxide synthase (nNOS). Although it was known that nNOS regulates the release of its product, the messenger molecule nitric oxide, all of the proteins involved in its cellular localization were previously unknown.

Mammalian Capon protein has the sequence disclosed in SEQ ID NO:2 or 4, 15 or other sequences which are at least about 80, 85, 87, 89, or 90% identical. Any biologically active variants of this sequence that may occur in mammalian tissues are within the scope of this invention. Biologically active variants bind to and inhibit nNOS binding to PSD95 and PSD93. Mammalian Capon proteins may comprise amino acids 1-503 as shown in SEQ ID NO:2 or 1-156 as shown in SEQ ID NO: 4. 20 Fragments of a mammalian Capon protein, comprising at least eight, nine, ten, twelve, thirteen, or sixteen consecutive amino acids selected from the sequence shown in SEQ ID NO:2 or 4, may also be used. Such fragments may be useful, for example, in various assays, as immunogens, or in therapeutic compositions. They may also be used as preparative reagents for purifying nNOS. A fusion protein may also be used for 25 many of these purposes, including as a reagent and as an immunogen.

A fusion protein consists of a full length mammalian Capon protein or a Capon protein fragment fused to a second protein or protein fragment by means of a peptide bond. The second protein or protein fragment may be, for example, a ligand for yet a third molecule. The second protein or protein fragment may be labeled with a detectable marker or may be an enzyme that will generate a detectable product. A 30

fusion protein may be useful, for example, to target full-length Capon protein or a Capon fragment comprising one or more specific domains, to a specific location in a cell or tissue.

Any of these Capon-related proteins may be produced by expressing Capon cDNA sequences in prokaryotic or eukaryotic host cells, using known expression vectors. Synthetic chemistry methods can also be used to synthesize Capon protein, fusion protein, or fragments. Alternatively, Capon protein can be extracted, using standard biochemical methods, from Capon-producing mammalian cells, such as brain cells. The source of the cells may be any mammalian tissue that produces Capon protein including human, rat, or mouse. Methods of protein purification, such as size exclusion chromatography, ammonium sulfate fractionation, ion exchange chromatography, affinity chromatography, or preparative gel electrophoresis, are well known in the art. Given the sequences disclosed in SEQ ID NO:2 and 4, an ordinary artisan can readily select appropriate methods to obtain a preparation of mammalian Capon protein that is substantially free from other mammalian proteins. An isolated Capon protein is purified from other compounds that may normally associate with Capon protein in a cell, such as certain proteins, carbohydrates, lipids, or subcellular organelles.

The present invention also provides a preparation of antibodies that specifically bind to mammalian Capon protein. The antibodies may be polyclonal or monoclonal and may be raised against biochemically isolated, chemically synthesized, or recombinantly produced full-length Capon protein, Capon protein fragments, or Capon fusion proteins. Techniques for raising antibodies directed against intracellular proteins such as mammalian Capon are well known in the art. The antibodies bind specifically to Capon epitopes, preferably epitopes not present on other mammalian proteins. Antibodies that bind specifically to Capon proteins include those that bind to full-length Capon protein, Capon fragments or degradation products, as well as to alternatively spliced forms of Capon proteins, or to Capon fusion proteins. In preferred embodiments of the invention the antibodies prevent Capon binding to nNOS, immunoprecipitate Capon protein from solution, and react with Capon protein on Western blots of polyacrylamide gels. Preferably the antibodies do not exhibit

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nonspecific cross-reactivity with other mammalian proteins on Western blots or in immunocytochemical assays. Techniques for purifying Capon antibodies are those which are available in the art. In a more preferred embodiment, antibodies are affinity purified by passing antiserum over a support column to which Capon protein is bound and then eluting the bound antibody, for example with high salt concentrations. Any such techniques may be chosen to achieve the preparation of the invention.

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The polynucleotides of the present invention encode Capon protein. These polynucleotides may be isolated and purified free from other nucleotide sequences by standard purification techniques, using restriction enzymes to isolate fragments comprising the Capon encoding sequences. The polynucleotide molecules are preferably intron-free and have the sequence shown in SEQ ID NO:1 or 3. Such Capon cDNA molecules can be made *inter alia* by using reverse transcriptase with Capon mRNA as a template. The polynucleotide molecules of the invention can also be made using the techniques of synthetic chemistry given the sequence disclosed herein. The degeneracy of the genetic code permits alternate nucleotide sequences to be synthesized that will encode the Capon amino acid sequence shown in SEQ ID NO:2 or 4. All such nucleotide sequences are within the scope of the present invention, as well as those which are at least 70, 75, 80, 85, or 90% identical. The Capon polynucleotide molecules can be propagated in vectors and cell lines as is known in the art. The constructs may be on linear or circular molecules. They may be on autonomously replicating molecules or on molecules without replication sequences. Recombinant host cells can be formed by introducing the genetic constructs of the present invention into cells. Any of those techniques which are available in the art can be used to introduce genetic constructs into the cells. These include, but are not limited to, transfection with naked or encapsulated nucleic acids, cellular fusion, protoplast fusion, viral infection, and electroporation. Introduction of genetic constructs may be carried out *in vitro* or *in vivo*.

The invention also provides a recombinant DNA construct for expressing Capon antisense nucleic acids. The construct contains a promoter and a coding sequence for Capon consisting of at least 12 and preferably at least 15 or 20 contiguous base pairs selected from SEQ ID NO:1 or 3. The Capon coding sequence

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is in an inverted orientation with respect to the promoter, so that when the sequence is transcribed from the promoter, an RNA complementary to native Capon-encoding mRNA is produced. The construct may also include a terminator at the 3' end of the inverted Capon coding sequence. The antisense molecules produced using the DNA construct of the invention may be used to decrease or prevent the transcription of Capon mRNA. The antisense molecules may be used *in vitro* or *in vivo*, as pharmacological agents for the purpose of influencing nNOS activity.

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According to the present invention, nNOS is inhibited by mammalian Capon protein, which competes with PSD95 and PSD93 for binding to nNOS, thereby inhibiting nNOS activity. Suitable inhibitory concentrations range from 1 nM to 1 mM. In a preferred embodiment the concentration of Capon protein is at least 250 nM. In a more preferred embodiment the concentration of Capon protein is at least 1  $\mu$ M. Greater concentrations of Capon protein may also be used. nNOS activity may be measured, for example, by assaying nitric oxide-dependent cGMP formation in HEK 293 cells cotransfected with DNA encoding Capon and nNOS. Other cell lines, such as mouse N1E-115 neuroblastoma cells, may be used as well. Formation of cGMP may be measured, for example, by radioimmunoassay or by spectrophotometry. nNOS activity may be measured in intact cells or in cell lysates. Other assays for measuring nNOS activity may also be used. NO or citrulline can also be measured.

The present invention also provides methods of screening test compounds for the ability to decrease or augment nNOS activity. The test compounds may be pharmacologic agents already known in the art or may be compounds previously unknown to have any pharmacological activity. The compounds may be naturally occurring or designed in the laboratory. They may be isolated from microorganisms, animals, or plants, and may be produced recombinantly, or synthesized by chemical methods known in the art. A test compound can be contacted with a mixture of mammalian Capon protein (or the NOS-binding portion thereof) and a polypeptide containing an nNOS PDZ domain which is a contiguous sequence selected from the N-terminal about 100 amino acids of nNOS amino acid sequences as shown in SEQ ID NO:5 and 6. Analogous domains in other mammalian nNOS proteins can also be used. These are referred to as biologically active, naturally occurring variants of the rat

or human protein. These molecules may be produced recombinantly or may be synthesized using standard chemical methods. The nNOS or Capon binding partner may consist of less than the entire nNOS. The two binding partners may be prebound prior to the step of contacting with the test compound. Alternatively, the test compound may contact one of the binding partners before the second binding partner is added. The PDZ domain-containing molecule may be in solution or may be bound to a solid support. These molecules may be unlabeled or labeled, for example, with a radioactive, fluorescent, or other detectable marker. They may be fusion proteins comprising a nNOS PDZ domain and another protein with or without a detectable enzymatic activity. The amount of at least one of the two binding partners that is bound or unbound in the presence of the test compound is then measured. A number of methods may be used to measure the amount of bound molecules. For example, the relative concentration of bound to unbound may be detected by examining the apparent molecular masses of the molecules by size exclusion chromatography or by polyacrylamide gel electrophoresis under non-reducing conditions. Other methods of measuring binding or dissociation of the molecules will readily occur to those of ordinary skill in the art and can be used. A test compound that decreases the amount of the polypeptide and Capon that are bound is a potential drug for increasing nNOS activity. A test compound that increases the amount of the polypeptide and Capon that are bound is a potential drug for decreasing nNOS activity.

According to the present invention a method is also provided of using the yeast two-hybrid technique to screen for test compounds that decrease or augment nNOS activity. The yeast two-hybrid technique is generically taught in Fields, S. and Song, O., *Nature* 340, 245-46, 1989. In a preferred embodiment, a cell is contacted with a test compound. The cell comprises a first fusion protein comprising a DNA binding domain and all or a portion of a mammalian Capon protein consisting of a contiguous sequence of amino acids selected from the amino acid sequence shown in SEQ ID NO:2 and capable of binding to nNOS (this typically requires the 13 carboxy terminal amino acids). The cell also comprises a second fusion protein comprising a transcriptional activating domain and all or a portion of nNOS, wherein the portion comprises a contiguous sequence of amino acids selected from amino acids 14-89 as

shown in SEQ ID NO:5 or 6 or naturally occurring biologically active variants thereof. Alternatively, the DNA binding domain and the transcriptional activating domains can be paired with the opposite proteins. The interaction of the portion of the Capon protein with the portion of nNOS reconstitutes a sequence specific transcriptional activating factor. A reporter gene is also present in the cell. The reporter gene comprises a DNA sequence to which the DNA binding domain of the first fusion protein specifically binds. When the Capon and nNOS regions are bound together, the DNA binding domain and the transcriptional activating domain will be in close enough proximity to reconstitute a transcriptional activator capable of initiating transcription of a detectable reporter gene in the cell. The expression of the reporter gene in the presence of the test compound is then measured. A test compound that increases the expression of the reporter gene is a potential drug for decreasing nNOS activity. A test compound that decreases the expression of the reporter gene is a potential drug for augmenting nNOS activity. Test compounds which increase nNOS activity are potential drugs for modulating aggressive behaviour, particularly aggressive sexual behavior. Test compounds which decrease nNOS activity are potential drugs for treating stroke patients and other neuronal degeneration which is mediated by NO.

Many DNA binding domains and transcriptional activating domains can be used in this system, including the DNA binding domains of GAL4, LexA, and the human estrogen receptor paired with the acidic transcriptional activating domains of GAL4 or the herpes virus simplex protein VP16 (See, e.g., G.J. Hannon *et al.*, *Genes Dev.* 7, 2378, 1993; A.S. Zervos *et al.*, *Cell* 72, 223, 1993; A.B. Votjet *et al.*, *Cell* 74, 205, 1993; J.W. Harper *et al.*, *Cell* 75, 805, 1993; B. Le Douarin *et al.*, *Nucl. Acids Res.* 23, 876, 1995). A number of plasmids known in the art can be constructed to contain the coding sequences for the fusion proteins using standard laboratory techniques for manipulating DNA (see, e.g., Example 1, below). Suitable detectable reporter genes include the *E. coli lacZ* gene, whose expression may be measured colorimetrically (see, e.g., Fields and Song, *supra*), and yeast selectable genes such as *HIS3* (Harper *et al.*, *supra*; Votjet *et al.*, *supra*; Hannon *et al.*, *supra*) or *URA3* (Le Douarin *et al.*, *supra*). Methods for transforming cells are also well known in the art. See, e.g., A. Hinnen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 75, 1929-1933, 1978. The test compound may

comprise part of the cell culture medium or it may be added separately. The tester cell need not be a yeast cell, but may be a bacterial, other fungal, or mammalian cell.

In another embodiment, a cell is contacted with a test compound. In this embodiment, the cell comprises (i) a first expression vector comprising a subgenomic polynucleotide encoding nNOS or a naturally occurring biologically active variant thereof, and (ii) a second expression vector comprising a subgenomic polynucleotide encoding a portion of Capon or a naturally occurring biologically active variant thereof. The portion of Capon is capable of binding to nNOS. NO production by the cell is then measured, for example by radioimmunoassay or by spectrophotometry. A test compound that increases the amount of NO produced by the cell is a potential drug for augmenting nNOS activity. A test compound that decreases the amount of NO in the cell is a potential drug for decreasing nNOS activity. NO production may be determined by assaying for cGMP or citrulline as well. A test compound which binds to nNOS at the Capon binding site may either inhibit Capon binding, thus favoring the interaction of nNOS and PSD95 or mimic Capon thus inhibiting the interaction of nNOS and PSD95.

Because expansion of glutamine repeats has been shown to be associated with neurodegenerative and other neurological diseases, the glutamine repeat in Capon is believed to be relevant to pathogenesis. Thus either the Capon protein or the gene encoding it can be examined for the number of glutamine residues in the glutamine repeat region, or the number of glutamine codons (CAG) in the CAG repeat region of the gene. Expansion to a number greater than 6 indicates a propensity for or the presence of a neurological disease, likely a neurodegenerative disease. As in other examples where this mechanism has been demonstrated, the degree of expansion is associated with the severity of disease. It is likely that the glutamine repeat region interacts with a binding partner, and the avidity of the interaction is governed by the degree of expansion of the glutamine repeats. Any means for determining the sequence of a gene or protein may be used, including but not limited to direct sequencing, hybridization with allele-specific probes, binding to antibodies, size determination on gel electrophoresis.

5        The main finding of this study is the identification of a novel protein, CAPON, which interacts selectively with nNOS. The interaction of CAPON with nNOS is highly specific and has been verified by several methods of monitoring protein-protein interactions. The similarities in neuronal localizations of CAPON and nNOS imply that these proteins interact physiologically and that the principal biological function of CAPON may be to interact with nNOS. The apparent selectivity of this interaction contrasts with other nNOS binding proteins such as PSD95, PIN and calmodulin, each of which bind to multiple other proteins.

10      The competitive binding for nNOS by CAPON and PSD95 suggests a model for regulating the translocation of nNOS between cytoplasm and synaptic structures (Figure 7). Presumably, NO release into the synaptic space must be preceded by its translocation to synaptic structures by binding to PSD95. We propose that this process can be blocked by CAPON's removal of nNOS from PSD95, and translocating nNOS into the cytoplasm, or some other cellular compartment. In this manner 15     CAPON could lead to effective nNOS inhibition. Although CAPON does not inhibit nNOS catalytic activity directly (data not shown), CAPON would reduce the accessibility of nNOS to NMDA receptor-mediated calcium influx, thus diminishing the capacity of nNOS to exert its physiologic or pathologic effects. Small molecules which specifically bind to nNOS in a manner similar to that of CAPON are useful for 20     blocking NO-mediated neuronal degeneration.

25      We have explored potential mechanisms that might regulate the nNOS/CAPON interaction. For instance, we phosphorylated nNOS in transfected HEK 293 cells by treatment with forskolin, phorbol esters, dibutyryl cyclic AMP and 8-bromo-cyclic GMP and in vitro or with purified nNOS protein utilizing protein kinase C, protein kinase A and calcium calmodulin dependent protein kinase using methods described previously (Bredt et al., 1992). We have been unable to alter CAPON-nNOS interactions by any of these treatments.

30      Conceivably, phosphorylation of CAPON regulates its interactions with nNOS. Recently some of us showed that phosphorylation of the n-2 serine in the potassium channel BIRK-2 regulates its binding to a PDZ domain in PSD95 (Cohen et al., 1996). CAPON, and several other PDZ-domain ligands (Songyang et al., 1997), lack a serine

in this position and so must be regulated in some other manner. One possible mechanism may be a regulation of the ligand's C-terminal secondary structure. A recent crystallographic study of a PDZ domain complexed with a short cognate peptide shows that the peptide binds in an antiparallel beta-sheet conformation, with characteristic beta-sheet contacts between the peptide and a strand of a beta-sheet within the PDZ domain (see Doyle et al., 1996). In a physiologic setting, the unbound cognate sequence may constitutively adopt a beta-sheet conformation, with the other beta-strands coming from other, possibly distant, residues within the ligand protein's sequence. This beta-sheet might constitute an endogenous high-affinity ligand. This notion is supported by our observation that short, presumably unfolded, peptides comprising the C-terminal nine residues of CAPON bind nNOS weakly, while 16-residue peptides are more potent competitors ( $IC_{50}=10$  mM), although both are much less effective than 100 amino acid fusion proteins which are active in the nanomolar range (see Figure 5). Peptide competitors that interact with other PDZ domains have also been utilized at 10 and 500 mM concentrations (see Brenman et al., 1996; Kornau et al., 1995). Conceivably, the nNOS-CAPON interaction would be disrupted simply by disrupting the beta-sheet conformation of the C-terminus, which might be achieved by phosphorylation at a distance.

The nNOS PDZ domain is the first example of a PDZ domain which binds to other PDZ domains. The region of nNOS which possesses this property is the PDZ domain plus the adjacent ~50 amino acids on the carboxyl-side of the PDZ domain (residues 1-150) (Brenman et al., 1996). The additional amino acids in this super-sized PDZ domain may be required to accommodate larger ligands such as other PDZ domains. The finding in this report of another physiologic ligand for the PDZ domain, namely, the C-terminal region of CAPON, raises the question of whether the same or different portions of the nNOS PDZ domain account for the binding to two seemingly different ligands. Because these interactions are mutually exclusive, it is likely that the ligand-binding cleft in the PDZ domain mediates both interactions. Previously identified proteins which contain C-terminal PDZ-binding sequences have been membrane associated. By contrast, CAPON is soluble. This demonstrates that PDZ domains may mediate purely cytosolic protein-protein interactions.

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Stricker et al. (1997) recently characterized the specificity of the nNOS PDZ-binding domain. These researchers used a phage display method to identify NOS-binding peptides. Peptides ending in the sequence aspartate-X-valine were found to be high affinity ligands. Interestingly, unlike CAPON, these peptides did not bind the canonical nNOS PDZ domain (amino acids 13-89) but bound the extended PDZ domain only (amino acids 1-150). This extended domain is the minimal sequence which mediates PDZ-PDZ interactions. Presumably the differences in the binding sites in nNOS for the phage display peptide and CAPON account for the differences between the sequence specificity requirements for PDZ-PDZ interactions and PDZ-CAPON interactions.

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The following are provided for exemplification purposes only and are not intended to limit the scope of the invention which has been described in broad terms above.

### **EXAMPLE 2.**

#### **Identification and Cloning of CAPON**

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We conducted a yeast two-hybrid screen employing the first 377 amino acids of nNOS, a region which includes the PDZ domain that comprises the first 100 amino acids of nNOS. Screening of six million clones resulted in the identification of three distinct cDNA inserts, one of which, PIN, has been previously reported (Jaffrey and Snyder, 1996) while the other two are overlapping cDNAs derived from a gene which is designated CAPON. The CAPON two-hybrid clones share a common carboxyl terminus and are predicted to translate into 125 and 327 amino acid peptides followed by a stop codon. The 125 amino acid C-terminal fragment of CAPON specifically interacts with nNOS in the two-hybrid system as is evident from the failure of CAPON to interact with fragments of PSD93 and PSD95 containing PDZ domains (Figure 1A). Moreover, nNOS fails to interact with another control protein, c-fos. To obtain a full-length CAPON cDNA, we screened a rat brain cDNA library with the larger two-hybrid clone and isolated a 2100 bp cDNA which overlapped with the two-hybrid clone and was used to assemble a final 2820 bp cDNA (see Methods). The conceptual translation of this cDNA produces a 503 amino acid protein (Figure 1B). The first ATG in the cDNA was 393 bp from the 5' end of the cDNA and was situated in a

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context that conformed to the Kozak consensus sequence for an initiator methionine (Kozak, 1991).

CAPON displays no significant homology to any other known class of protein except for an N-terminal 145 amino acid stretch of amino acids which has residues suggestive of a phosphotyrosine-binding (PTB) domain (Zhou et al., 1995). CAPON's PTB domain most closely resembles the mouse numb protein's PTB domain (Zhong et al., 1996) with nearly 28% sequence identity on this region. The similarity between CAPON and numb are limited to this domain. PTB domains are targetted to phosphotyrosine containing proteins such as growth factor receptors (reviewed in van der Geer and Pawson, 1995).

Outside of the PTB domain, CAPON lacks any well known consensus sequences except for an 18 nucleotide stretch of CAG repeats that corresponds to six glutamines. Glutamine repeats occur in proteins whose expansion results in neurodegenerative diseases as exemplified by huntingtin, the protein which is altered in Huntington's disease (Ross, 1995). A BLAST search (Altschul et al., 1990) of an expressed sequence-tag database, dBEST, reveals a human brain-derived EST with ~75% nucleotide identity to CAPON. The cDNA insert was 1.4 kb and corresponds to the C-terminal 156 amino acids of CAPON plus one kb of 3' UTR. The conceptual translation of this portion of human CAPON has 92% amino acid identity with the rat protein (Figure 1B).

Northern (RNA) blotting reveals a predominant 7.5 kb transcript which is detected only in brain regions with no expression evident in adrenal, bladder, heart, kidney, lung and skeletal muscle (Figure 1C). Marked regional variations occur in the brain with highest densities in the cerebral cortex and medulla-oblongata and lowest levels in the hippocampus.

To assess the specificity of interactions between CAPON and nNOS, we evaluated the binding of a GST-CAPON fusion protein, consisting of C-terminal 125 amino acids of CAPON, with nNOS, eNOS and inducible NOS (iNOS) (Figure 2A). Lysates of HEK-293 cells transfected with expression plasmids for each of the three forms of NOS were incubated with GST-CAPON. After extensive washing of the resin, bound nNOS was detected by Western blotting with the appropriate isoform-specific

antibody...nNOS interacts strongly with GST-CAPON, while eNOS and iNOS do not interact. No interactions are evident with the GST control.

We also examined interactions of CAPON and nNOS by utilizing a GST-fusion protein of the NH<sub>3</sub>-terminal 100 amino acids of nNOS, the PDZ domain. Following incubation with cerebellar lysates the washed GST-nNOS resin was subjected to SDS-PAGE and Western blotted with a purified antibody directed against the C-terminal 125 amino acids of CAPON. A single band corresponding to CAPON is detected, while no CAPON is bound to the GST control. A lower molecular weight band in homogenates is occasionally detected using our anti-CAPON antibodies. This protein fails to interact with GST-nNOS fusion proteins (Figure 2B) and may represent an unrelated cross-reactive protein, or the product of an alternatively spliced CAPON mRNA which fails to bind nNOS.

The protein-protein interactions we detected with cell lysates and GST-fusion proteins might have reflected a tertiary interaction between nNOS, an unidentified protein and CAPON. To determine if CAPON directly binds to nNOS, we conducted blot overlay experiments (Figure 2C). Lysates of HEK 293 cells transfected with nNOS were resolved on a polyacrylamide gel, transferred to nitrocellulose and then probed with [<sup>32</sup>P]GST-CAPON. The radiolabeled CAPON probe binds to a single 160 kD band comigrating with a nNOS standard, while no binding is evident in mock transfected cells, demonstrating that CAPON physically interacts in a direct manner with nNOS.

To ascertain if complexes of CAPON and nNOS exist physiologically, we used two approaches. Our first approach took advantage of a NOS-affinity resin consisting of 2', 5'-ADP ribose crosslinked to an agarose matrix. This resin has been used previously to purify nNOS from cerebellar supernatants (Bredt and Snyder, 1990). Following incubation with cerebellar supernatants, the resin and washed extensively. As expected, a significant portion of the nNOS found in the starting material was bound to the resin (Figure 2D). To determine if nNOS and CAPON were physiologically associated, we next assayed for CAPON bound to the resin. Like nNOS, CAPON was substantially enriched in the bound fraction. As a control we assayed for the resin-binding ability of CAPON from supernatants derived from mice

with a genomic deletion of nNOS. These mice express a truncated version of nNOS which lacks the PDZ domain and is unable to bind CAPON. Substantially less CAPON in these supernatants bound to the 2', 5'-ADP ribose resin indicating that CAPON has negligible intrinsic affinity for the resin and that CAPON-binding in wild-type supernatants was due to nNOS. The smaller cross-reactive band was also enriched on this resin, supporting the notion that it is in some manner related to CAPON by alternative splicing or proteolytic degradation. Interestingly, the total level of CAPON in knockout supernatants was approximately one half that in wild-type mice, possibly due to the absence of a stabilizing effect of nNOS.

As a second approach, we immunoprecipitated CAPON from cerebellar supernatants and assayed for nNOS by Western blot (Figure 2E). nNOS coprecipitates with anti-CAPON antibodies but is not detected in immunoprecipitates generated using preimmune serum, an antibody to the G-protein subunit  $\beta 1$ , or with an antibody to cyclin-dependent kinase 2. To determine if the enrichment observed in anti-CAPON immunoprecipitates was specific, we asked if a control protein, protein kinase C- $\beta$  I/II, was similarly enriched in these fractions. We found that this protein was absent in all of the precipitates (Figure 2E) indicating that the coprecipitation of nNOS with CAPON was specific. These two approaches support the notion that CAPON and NOS exist as a complex in the rat cerebellum.

In other experiments we metabolically labeled NIE-115 mouse neuroblastoma cells with [ $^{35}$ S] methionine and examined for the presence of proteins that would bind to GST-CAPON. The only protein that specifically interacted with CAPON is a 160 kD protein that comigrates with an nNOS standard (unpublished observations), suggesting that nNOS is the most abundant CAPON-binding protein in these cells.

#### 25 General Methods and Materials

Molecular biology reagents were from New England Biolabs (Beverly, MA) and all other reagents were from Sigma (St. Louis, MO) except as indicated. Protein concentrations were determined by Bradford assay.

#### Yeast two-hybrid methods

30 Two-hybrid screens and the construction of the parent vectors pPC86, containing the GAL4-activation domain, and pPC97, containing the GAL4-DNA binding domain,

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have been described (Jaffrey and Snyder, 1996). Plasmid pBD-NOS(2-377) was prepared by the insertion of an nNOS-PCR product corresponding to amino acids 2-377 of rat nNOS into the Sal I and Bgl II sites of pPC97, resulting in an open reading frame encoding a GAL4 BD-NOS fusion protein (Jaffrey and Snyder, 1996). The nNOS fragment was constructed by PCR using the following primers: 5'-GACTAGTCGACTGAAGAGAACACGTTGGG-3' (coding strand, SEQ ID NO: 7) and 5'-TCTGCAGATCTCAGTGGGCCTTGGAGCCAAA-3' (noncoding strand. SEQ ID NO: 8).

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A rat hippocampal cDNA library in pPC86 (Li et al., 1995) was amplified once in DH10B (Gibco BRL) as described (Jaffrey and Snyder, 1996) and transformed into yeast containing the pBD-NOS(2-377) plasmid. pAD-CAPON1 and pAD-CAPON2 were identified as 0.8 kb and 1.9 kB clones, respectively, that activated lacZ transcription and conferred histidine prototrophy in the presence of pBD-NOS(2-377). Plasmids were sequenced by automated fluorescent sequencing of both strands. Yeast two-hybrid vectors containing the second PDZ domain of PSD93 (amino acids 116-421) and the three PDZ domains of PSD95 (amino acids 20-364) have been described previously (Brenman et al., 1996).

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Truncated NOS fragments comprising amino acids 2-165 and 2-284 were generated by restriction of the initial NOS (2-377) PCR fragment with Nco I and Ava I, respectively followed by Klenow-filling in of that end and ligation into pPC97. Other truncated NOS fragments were prepared by PCR and have been described (Jaffrey and Snyder, 1996).

#### cDNA cloning of CAPON

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A CAPON DNA probe was generated by the random hexamer method using the pAD-CAPON2 cDNA as a template. This probe was used to screen a rat brain lambdaZAP II cDNA library (Stratagene) using methods described by the manufacturer. A 2.1 kb cDNA was isolated which overlapped with the pAD-CAPON2 clone. The cDNAs were ligated at an overlapping Xba I site to produce the full-length 2,812 bp cDNA and subcloned into pCMV for eukaryotic expression. The human CAPON EST was the sole CAPON homolog identified in a BLAST search (Altschul et al., 1990).

I.M.A.G.E. consortium (<http://www-bio.llnl.gov/bbrp/image/image.html>) clone 34183 (Lennon et al., 1996) was purchased from Research Genetics (Huntsville, AL).

#### RNA (Northern) blotting

30 micrograms of whole RNA were isolated from rat tissues using the Triazol reagent (Gibco BRL) and separated on agarose-formaldehyde gels. RNA was transferred to Hybond N+ membranes (Amersham) and a DNA probe, generated using the random hexamer method with the two-hybrid CAPON cDNA as a template, was hybridized in Rapid-hyb buffer (Amersham) overnight at 65°C. The blots were subsequently washed sequentially in 2X SSC with 0.1% SDS, once at room 5 temperature for 15 min, 1X SSC with 1% SDS twice at 65°C, 0.1XSSC with 1% SDS twice at 65°C, and then with 0.1X SSC with 5% SDS twice at 65°C. The blot was apposed to film for 4 days at -80°C to visualize the bands.

#### GST-fusion protein binding assays

10 GST-fusion proteins were prepared in BL21(DE3) Escherichia coli (Novagen) with glutathione agarose as an affinity resin for purification (Smith and Johnson, 1988), except that bacterial pellets were sonicated in lysis buffer (50 mM Tris-HCl (pH 7.7), 15 100 mM NaCl, and 2 mM EDTA), supernatants were adjusted to 1% Triton X-100, washes were done in 50 mM Tris-HCl (pH 7.7), 500 mM NaCl, 2 mM EDTA, and 1% Triton X-100, and protein was purified with elution buffer (50 mM tris-HCl (pH 7.7), 20 100 mM NaCl, 10 mM reduced glutathione, and 2 mM EDTA).

Transfections were performed with 10 µg of each plasmid using the calcium phosphate method. Following transfection, cells were sonicated in buffer A [50 mM Tris-HCl (pH 7.7), 100 mM NaCl, 2 mM EDTA, and 1% Triton X-100] and cleared by centrifugation. This cellular lysate was incubated with GST-fusion protein immobilized on glutathione-agarose for one hour at 4°C and washed extensively in HNTG buffer [20 mM Hepes (pH 7.4), 500 mM NaCl, 10% glycerol, and 0.1% Triton 25 X-100] five times, for ten minutes per wash at room temperature. A GST-CAPON fusion protein consisting of amino acids 379-503 was used for binding assays because it was more soluble when expressed in bacteria than larger CAPON fusion proteins.

For quantitative binding experiments, transfected cells were metabolically labeled overnight with 200 mci [<sup>35</sup>S] methionine and nNOS was purified by NADPH elution of 2',5' ADP ribose as described previously (Bredt and Snyder, 1990).

5 The material remaining on the resin was eluted with SDS-PAGE sample buffer and nNOS was detected by immunoblot using antibodies specific to each NOS isoform (Transduction Labs). A polyclonal antiserum to CAPON was generated in rabbits by using a His<sub>6</sub>-tagged CAPON fusion protein. GST-CAPON was crosslinked to glutathione agarose with dimethylpimelimidate and this resin was used to purify CAPON antibody. To confirm the specificity of the antibody, immune serum was 10 incubated with His<sub>6</sub>-CAPON which results in the abolishment of the signal. Incubation with His<sub>6</sub>-FKBP has no effect on the signal (data not shown).

15 For blot-overlay analysis, CAPON was inserted into pGEX-4T2, a derivative of PGEX4T2 in which two cyclic AMP-dependent protein kinase (PKA) sites were inserted between the GST moiety and the multiple cloning site (Jaffrey and Snyder, 1996). Kinase reactions and blot overlays were performed as described (Kavanaugh and Williams, 1994).

#### EXAMPLE 2.

##### **The C-terminus of CAPON Binds to the PDZ Domain of nNOS**

20 To examine the region of nNOS that binds to CAPON, we conducted yeast two-hybrid experiments with various truncations of nNOS (Figure 4A). As little as the first 100 amino acids of nNOS binds to the C-terminal 125 amino acids of CAPON. This portion of nNOS contains the full PDZ domain as defined by MacKinnon and associates (Doyle et al., 1996) who identified the PDZ consensus domain in nNOS as 25 amino acids 14-89. Deletion of the first 20 amino acids of nNOS, which includes the first seven amino acids of the PDZ domain, does not abolish binding, but larger NH<sub>3</sub>-terminal deletions abolish binding, presumably because they result in a loss of important structural components of the PDZ domain. The nNOS construct comprising amino acids 163-245, which represents the PIN-binding domain of nNOS (Jaffrey and Snyder, 1996), shows no interaction with CAPON.

30 PDZ domains typically interact with a characteristic C-terminal motif in other proteins (Songyang et al., 1997). By contrast, the nNOS PDZ domain binds directly

to other PDZ domains, such as those in PSD95, but has not previously been reported to interact with any known physiological C-terminal peptide motifs. Since we could not detect any PDZ domain motifs in the CAPON sequence we sought to determine the region in CAPON which accounted for nNOS binding. We investigated the domain of CAPON that interacts with nNOS using GST-CAPON fusion proteins containing various deletions at the C-terminus (Figure 4B). Immobilized fusion proteins were incubated with HEK 293 lysates containing nNOS. Bound nNOS was detected by Western blot. Robust interactions with nNOS are evident with constructs containing as little as the C-terminal thirteen amino acids of CAPON. Deleting the C-terminal 20 amino acids of CAPON abolishes its interactions with nNOS. These data show that the C-terminal portion of CAPON is necessary and sufficient for nNOS binding.

Recently Cantley and associates (Songyang et al., 1997) identified consensus sequences for binding to several PDZ domains. Binding of PDZ ligands involves the C-terminus of proteins, with determinants of specificity lying within the eight or fewer C-terminal amino acids. A consistent requirement among all the PDZ domain ligands is a hydrophobic residue, such as valine or leucine, as the final amino acid. To determine if the binding of CAPON to nNOS exhibits similar sequence-specificity, we examined the binding of mutagenized His<sub>6</sub>-CAPON fusion proteins to immobilized GST-nNOS PDZ domain fusion proteins (Figure 4C). The C-terminal residue of CAPON is a valine, and conversion of this residue to alanine abolishes binding. Binding is also greatly reduced by changing the penultimate amino acid from alanine to aspartate. However, changing the n-2 amino acid from isoleucine to serine or alanine does not alter binding. These experiments indicate that the nNOS-CAPON interaction resembles those of other PDZ-C-terminal peptide ligand interactions. Specifically, C-terminal residues of CAPON are important for the specificity in CAPON binding to nNOS.

#### **EXAMPLE 2.**

#### **CAPON and PSD95 Compete for Binding to nNOS**

Since the nNOS PDZ domain is capable of both PDZ-PDZ interactions and PDZ-C-terminal peptide interactions we wondered whether CAPON and PSD95 can

bind simultaneously to nNOS or whether their interactions with nNOS are mutually exclusive. To answer this question we incubated lysates of HEK-293 cells containing nNOS with various concentrations of a His<sub>6</sub>-CAPON fusion protein, comprising the last 125 amino acids of CAPON, and then added these lysates to GST-PSD95 immobilized on glutathione agarose resin (Figure 5A). After extensive washing of the resin, we assayed for nNOS bound to PSD95 by Western blot. As little as 5 nM His<sub>6</sub>-CAPON causes a substantial reduction of nNOS binding to GST-PSD95. Half maximal reduction of binding is evident between 5 and 50 nM His<sub>6</sub>-CAPON (Figure 5B). Deletion of the C-terminal 20 amino acids of CAPON abolishes its ability to serve as a competitor for nNOS binding to GST-PSD95. As controls, we examined the effects of 5  $\mu$ M His<sub>6</sub>-PIN or 5 mM His<sub>6</sub>-FK506 binding protein-12 kD (FKBP). Neither of these proteins compete for binding to nNOS. nNOS also binds to the second PDZ domain of PSD93, a protein which is highly homologous to PSD95 (Brenman et al., 1996). His<sub>6</sub>-CAPON is an effective competitor for nNOS binding to immobilized GST-PSD93 as well (Figure 5A, B). These effects of CAPON likely reflect its binding to nNOS rather than to the PDZ domains of either PSD95 or PSD93 because CAPON fails to interact with either PSD93 or PSD95 using (i) a two-hybrid assay (see Figure 1A) and (ii) in vitro experiments utilizing immobilized GST-PSD95 and purified recombinant CAPON (data not shown).

We wanted to determine if CAPON and PSD95 compete for binding to nNOS in intact cells. Accordingly, we transfected HEK-293 cells with various mixtures of expression plasmids containing cDNAs of hemagglutinin antigen (HA)-tagged nNOS, myc-tagged PSD95 and/or full-length CAPON. Following immunoprecipitation with antibodies to HA, we examined which proteins coprecipitated. In cells expressing HA-nNOS and myc-PSD95, antibodies to HA coprecipitate myc-PSD95 (Figure 6). When various amounts of CAPON cDNA containing expression plasmids are also transfected, HA-immunoprecipitates contain CAPON but substantially less PSD95.

### Immunoprecipitations

Immunoprecipitations were performed by homogenizing one rat cerebellum in 3 ml lysis buffer followed by centrifugation at 100,000  $\times$  g for 30 min at 4°C. Two hundred

microliters of the supernatant was incubated with 40 ml of protein A-agarose (Oncogene Sciences, Cambridge, MA) and 5 mg of the indicated antibody for 60 min at 4°C. The resins were then washed with IP wash buffer (50 mM Tris-HCl (pH 7.7), 400 mM NaCl, and 2 mM EDTA) six times and eluted in 60 ul of 1X SDS-PAGE sample buffer by boiling. Western blots were performed using an nNOS-specific monoclonal antibody (Transduction Labs) or a PKC- b I/II monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The immunoprecipitating antibodies used as controls were from Santa Cruz Biotechnology, Inc.

For experiments utilizing 2', 5', ADP sepharose (Pharmacia), tissues were prepared identically as for immunoprecipitations except mouse cerebella were used and the homogenization volume was 400 ml per cerebellum. Supernatants were incubated with 100 ul of affinity resin. Incubations and washes were performed identically as for immunoprecipitations. The generation of mice with a targetted deletion of nNOS has been described previously (Huang et al., 1993).

### 15 Immunohistochemistry

Adult Sprague-Dawley rats (200-250 gm) were obtained from Charles River and housed at the Johns Hopkins Animal Care Facility. A polyclonal antiserum to the C-terminal region of human nNOS (residues 1419-1433) was kindly provided by J. Spangenberg (IncStar, Stillwater, MN). The peroxidase Elite staining kit was from Vector Laboratories.

Anesthetized rats were perfused through the left ventricle with 50 ml of 0.9% NaCl followed by 500 ml 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed, cut into saggital blocks, and postfixed in 4% paraformaldehyde in 0.1 M PB for 4 h at room temperature. Blocks were cryoprotected for 2 days at 4°C in 50 mM sodium phosphate, pH 7.4/0.1 M NaCl/20% (vol./vol.) glycerol. Brain sections, 40 mm thick, were cut on a sliding microtome. Free-floating sections were incubated in PBS (10 mM, pH 7.4/0.19 M NaCl), containing 4% normal goat serum (Jackson Labs), and 0.2% Triton X-100 for 45 min, and then incubated overnight at 4°C with the primary antiserum diluted 1:500 (CAPON) or 1:15,000 (nNOS) in phosphate buffered saline (PBS) containing 2% goat

5

serum and 0.1% Triton X-100. Immunoreactivity was visualized with the Vectastain ABC Elite kit following the nickel-enhanced diaminobenzidine procedure. To test immunohistochemical specificity of the CAPON antiserum, the antiserum was incubated overnight with 13.5 mg/ml of the antigenic fusion protein before incubation with brain sections.

10

### In situ hybridization

In situ hybridization used DNA oligonucleotide probes corresponding to amino acids 478-503. Probes were end-labelled with [ $\alpha$ -<sup>32</sup>P] dATP and terminal deoxynucleotidyl transferase to a specific activity of 800 mCi/mg and in situ hybridization was performed as described previously (Jaffrey et al., 1994). Non-specific hybridization was determined using the corresponding sense probe.

15

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**SEQUENCE LISTING SUMMARY**

10 SEQ ID NO: 2. Rat capon cDNA  
SEQ ID NO: 2. Rat capon amino acids  
SEQ ID NO: 2. Human capon cDNA  
SEQ ID NO: 2. Human capon amino acids  
SEQ ID NO: 2. Rat nNOS amino acids  
SEQ ID NO: 2. Human nNOS amino acids  
15 SEQ ID NO: 2. nNOS probe  
SEQ ID NO: 2. nNOS probe

**CLAIMS**

1. An isolated mammalian Capon protein which binds to the PDZ domain of a mammalian nitric oxide synthase (nNOS).
2. The Capon protein of claim 1 which has the amino acid sequence shown in SEQ ID NO:2 or 4, and naturally occurring biologically active variants thereof.
3. The Capon protein of claim 1 which is made by isolating the protein from human cells.
4. The Capon protein of claim 1 which is made by isolating the protein from rat cells.
5. The Capon protein of claim 1 which is produced recombinantly.
- 10 6. The Capon protein of claim 1 which is produced by synthetic chemical methods.
- 15 7. A fusion protein which comprises a first protein segment and a second protein segment fused to each other by means of a peptide bond, wherein the first protein segment consists of at least eight contiguous amino acids selected from the amino acid sequence shown in SEQ ID NO:2 or 4.
8. The fusion protein of claim 7 wherein the second protein segment consists of glutathione-S-transferase.
9. An isolated polypeptide which consists of at least eight contiguous amino acids of Capon as shown in SEQ ID NO:2 or 4, wherein the polypeptide binds to a PDZ domain of a mammalian neuronal nitric oxide synthase (nNOS).
- 20 10. A preparation of antibodies which specifically bind to a Capon protein as shown in SEQ ID NO:2 or 4.
11. The preparation of antibodies of claim 10 wherein the antibodies are monoclonal.
- 25 12. The preparation of antibodies of claim 10 wherein the antibodies are purified from an animal antiserum.
13. The preparation of antibodies of claim 10 wherein the antibodies are affinity purified.

14. A subgenomic polynucleotide which encodes a Capon protein as shown in SEQ ID NO:2 or 4.
15. The subgenomic polynucleotide of claim 14 which is intron-free.
16. The subgenomic polynucleotide of claim 15 which comprises the sequence shown in SEQ ID NO:1 or 3.
- 5 17. A vector comprising the polynucleotide of claim 14.
18. A vector comprising the polynucleotide of claim 15.
19. A vector comprising the polynucleotide of claim 16.
20. A recombinant host cell which comprises the vector of claim 17.
- 10 21. A recombinant host cell which comprises the vector of claim 18.
22. A recombinant host cell which comprises the vector of claim 19.
23. A recombinant DNA construct for expressing Capon antisense nucleic acids, comprising:
  - a promoter; and
  - 15 a coding sequence for Capon consisting of at least 12 contiguous base pairs selected from SEQ ID NO:1 or 3, wherein the coding sequence is in an inverted orientation with respect to the promoter, such that upon transcription from said promoter an RNA is produced which is complementary to native mRNA encoding Capon.
- 20 24. The construct of claim 23 further comprising a transcription terminator, wherein the coding sequence is between the promoter and the terminator.
- 25 25. A method of inhibiting the activity of a mammalian neuronal nitric oxide synthase (nNOS), comprising the step of:
  - contacting a nNOS with a Capon protein having an amino acid sequence as shown in SEQ ID NO:2 or 4 or a naturally occurring biologically active variant thereof, wherein the Capon protein is present in an inhibitory-effective amount.
26. The method of claim 25 wherein said Capon protein is present at a concentration of at least 250 nM.
- 30 27. The method of claim 25 wherein the Capon protein is present at a concentration of at least 1  $\mu$ M.

28. A method of screening test compounds for the ability to decrease or augment nNOS activity, comprising the steps of:

5 (a) contacting a test compound with all or a portion of a mammalian Capon protein and a polypeptide comprising an nNOS PDZ domain, wherein the Capon protein is capable of binding to nNOS; and

10 (b) measuring the amount of Capon or the polypeptide that is bound or unbound in the presence of the test compound, a test compound that decreases the amount of bound Capon or the polypeptide being a potential drug for increasing nNOS activity, and a test compound that increases the amount of the polypeptide or Capon that are bound being a potential drug for decreasing nNOS activity.

29. The method of claim 28 wherein the test compound is contacted with at least one of the polypeptide and Capon prior to the step of contacting.

30. The method of claim 28 wherein one of the polypeptide and Capon is bound to a solid support.

15 31. The method of claim 28 wherein at least one of the polypeptide and Capon is radiolabeled.

32. The method of claim 28 wherein at least one of the polypeptide and Capon is a fusion protein.

33. The method of claim 28 wherein at least one of the polypeptide and Capon is a fusion protein that has a detectable enzyme activity.

20 34. A method of screening test compounds for the ability to decrease or augment nNOS activity, comprising the steps of:

(a) contacting a cell with a test compound, wherein the cell comprises:

25 i) a first fusion protein comprising (1) a DNA binding domain or a transcriptional activation domain, and (2) all or a portion of a mammalian Capon protein, wherein the portion consists of a contiguous sequence of amino acids selected from the amino acid sequence shown in SEQ ID NO:2 or 4, wherein the portion is capable of binding to nNOS;

30 ii) a second fusion protein comprising (1) a transcriptional activation domain or a DNA binding domain and (2) all or a portion of nNOS, wherein the portion comprises a PDZ domain, or a naturally occurring biologically active variant

thereof, wherein the interaction of the portion of the Capon protein with the portion of nNOS reconstitutes a sequence-specific transcriptional activating factor, wherein when the first fusion protein comprises a DNA binding domain the second fusion protein comprises a transcriptional activation domain and when the first fusion protein comprises a transcriptional activation domain the second fusion protein comprises a DNA binding domain; and

iii) a reporter gene comprising a DNA sequence to which the DNA binding domain of the first fusion protein specifically binds; and

(b) measuring the expression of the reporter gene, a test compound that increases the expression of the reporter gene being a potential drug for decreasing nNOS activity, and a test compound that decreases the expression of the reporter gene being a potential drug for augmenting nNOS activity.

35. A method of screening for drugs with the ability to decrease or augment nNOS activity comprising the steps of:

15 (a) contacting a cell with a test compound, wherein the cell comprises:

(i) a first expression vector comprising a subgenomic polynucleotide encoding at least the PDZ domain of nNOS or a naturally occurring biologically active variant thereof;

20 (ii) a second expression vector comprising a subgenomic polynucleotide encoding at least the portion of Capon or a naturally occurring biologically active variant thereof, wherein the portion of Capon is capable of binding to nNOS; and

(b) measuring the amount of nitric oxide produced by the cell, a test compound that increases the amount of nitric oxide being a potential drug for augmenting nNOS activity, and a test compound that decreases the amount of nitric oxide being a potential drug for decreasing nNOS activity.

36. A method of diagnosing a neurological disease or propensity for a neurological disease, comprising:

30 determining number of glutamine repeats present in a Capon protein of a patient wherein a number greater than 6 indicates a neurologic disease or a propensity therefor.

37. A method of diagnosing a neurological disease or a propensity for a neurological disease, comprising:

determining number of CAG repeats in a *Capon* gene of a patient, wherein a number greater than 6 indicates a neurologic disease or a propensity therefor.

5 38. A cell comprising one or more recombinant constructs, comprising:

(i) a first expression vector comprising a subgenomic polynucleotide encoding at least the PDZ domain of nNOS or a naturally occurring biologically active variant thereof;

10 (ii) a second expression vector comprising a subgenomic polynucleotide encoding at least a portion of Capon or a naturally occurring biologically active variant thereof, wherein the portion of Capon is capable of binding to nNOS.

15 39. A cell comprising one or more recombinant constructs, comprising:

i) a nucleotide construct encoding a first fusion protein comprising (1) a DNA binding domain or a transcriptional activation domain, and (2) all or a portion of a mammalian Capon protein, wherein the portion consists of a contiguous sequence of amino acids selected from the amino acid sequence shown in SEQ ID NO:2 or 4, wherein the portion is capable of binding to nNOS;

20 ii) a nucleotide construct encoding a second fusion protein comprising (1) a transcriptional activation domain or a DNA binding domain and (2) all or a portion of nNOS, wherein the portion comprises a PDZ domain, or a naturally occurring biologically active variant thereof, wherein the interaction of the portion of the Capon protein with the portion of nNOS reconstitutes a sequence-specific transcriptional activating factor, wherein when the first fusion protein comprises a DNA binding domain the second fusion protein comprises a transcriptional activation domain and when the first fusion protein comprises a transcriptional activation domain the second fusion protein comprises a DNA binding domain; and

25 30 iii) a reporter gene comprising a DNA sequence to which the DNA binding domain of the first fusion protein specifically binds, wherein upon reconstitution of the sequence specific transactivating factor, expression of the reporter gene is increased.

1 / 7  
A

GAL4-AD fusion

GAL4-BD fusion

240512-311

PSD31

PSD35

GAL4-AD fusion

CAPON

c-FOS

8

C

GAPDH   

Figure 1. Cloning of the CAPON cDNA and Distribution of CAPON mRNA

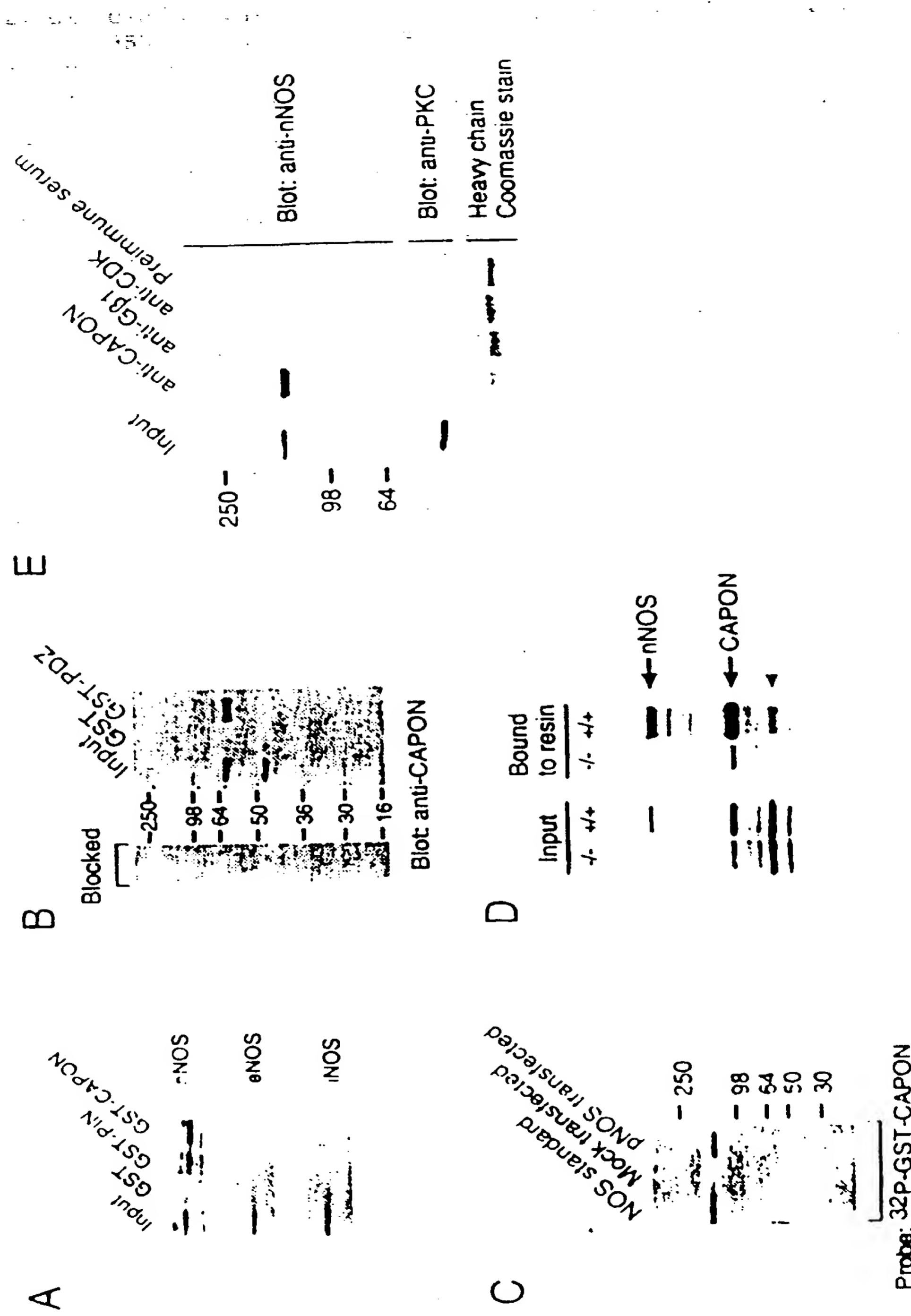
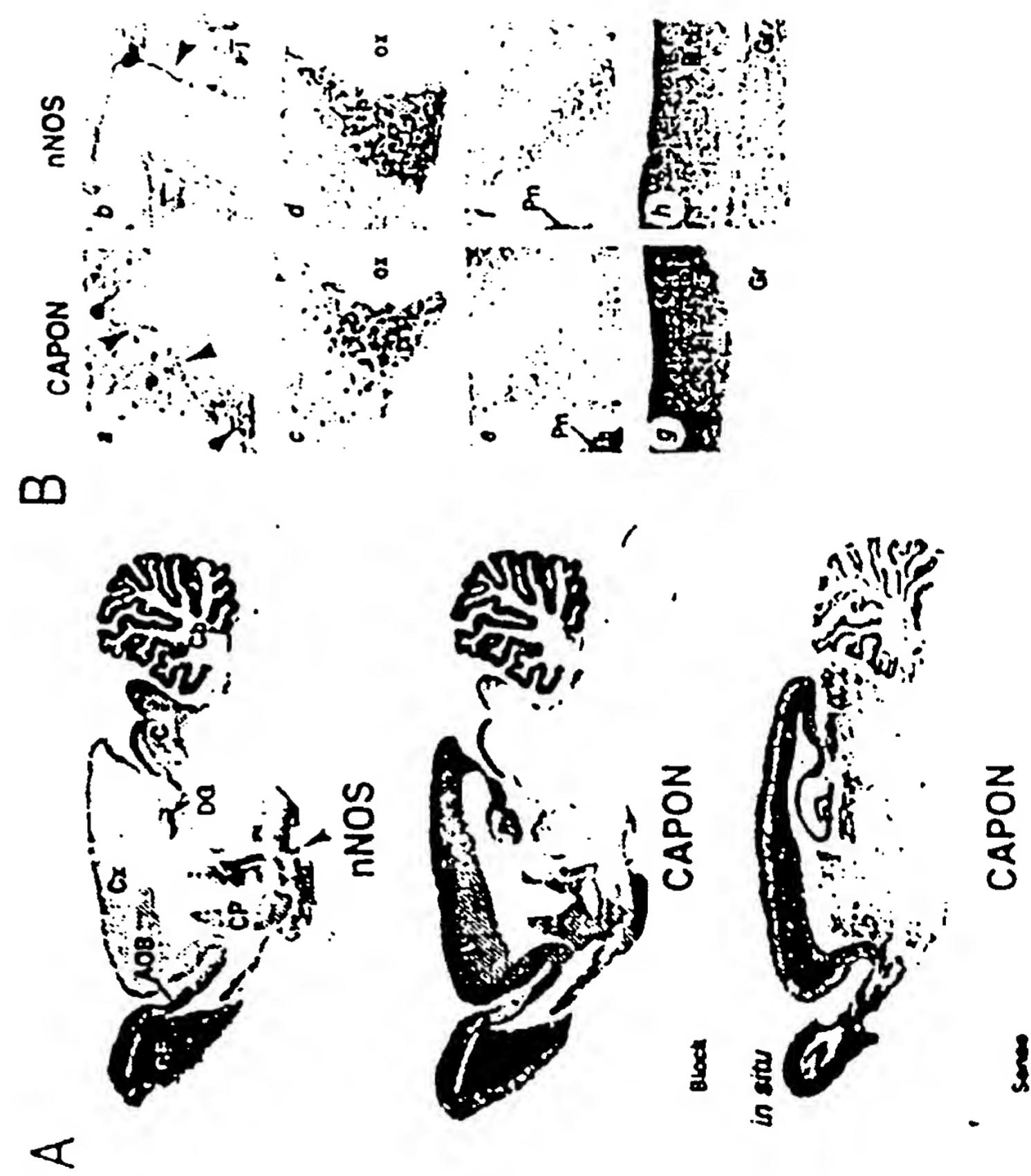


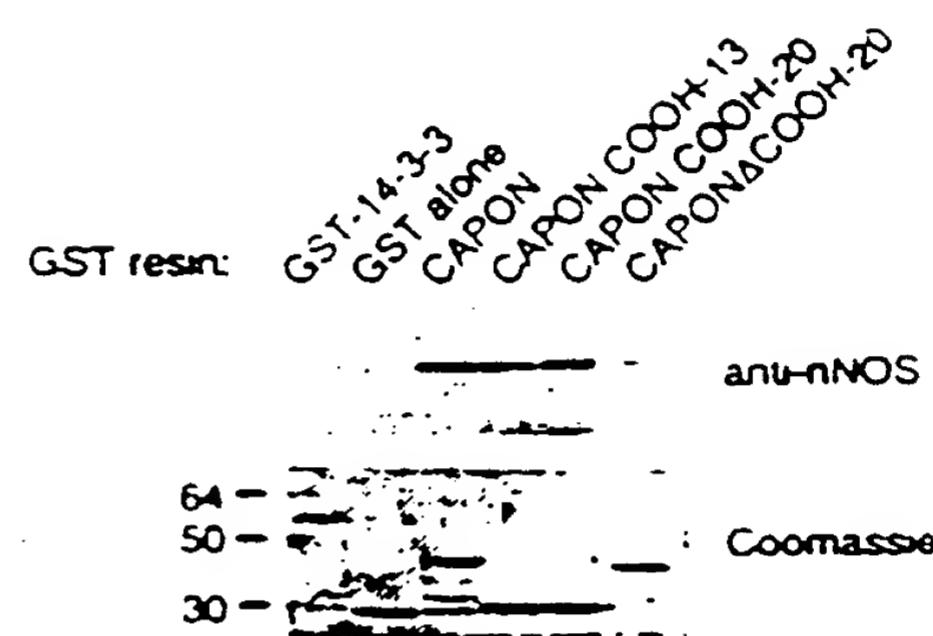
Figure 3. Immunohistochemical Localization  
of nNOS and CAPON



A

nNOS residues	Reporter
1-377	+
20-245	+
1-160	+
1-100	+
40-160	-
130-232	-
63-245	-

B



C

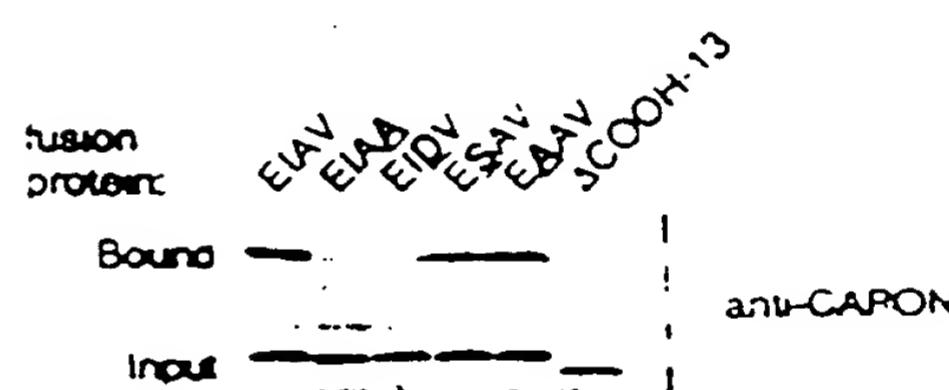
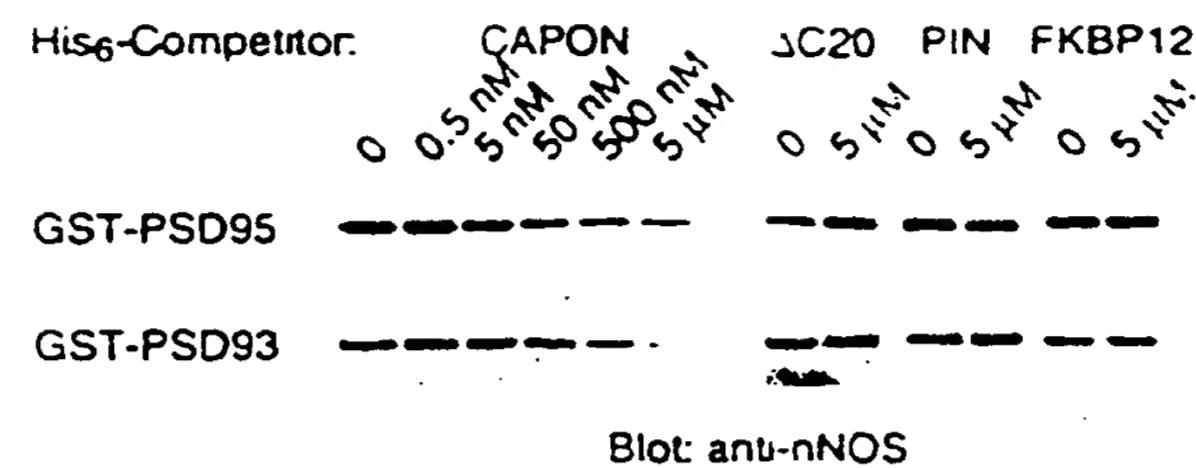


Figure 4. The PDZ Domain of nNOS Interacts with the C Terminus of CAPON

A



B

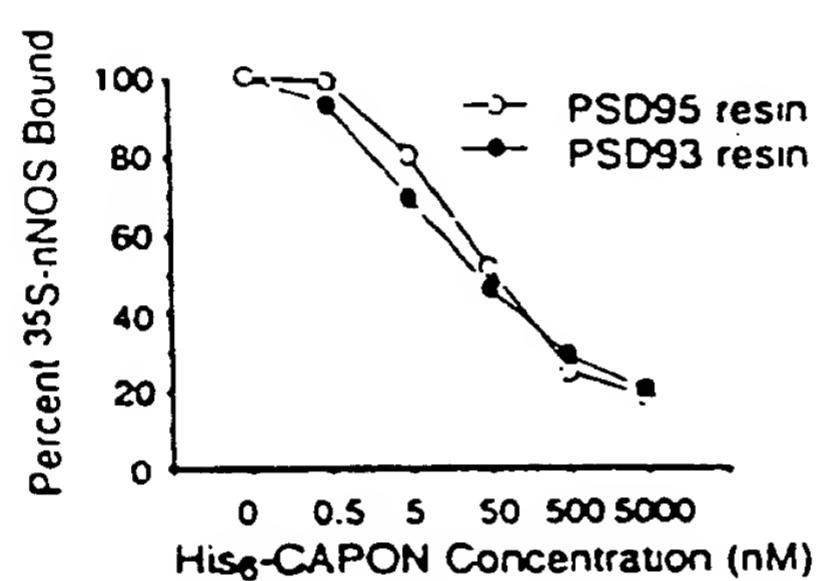


Figure 5. CAPON and PSD95 Compete for Binding to nNOS

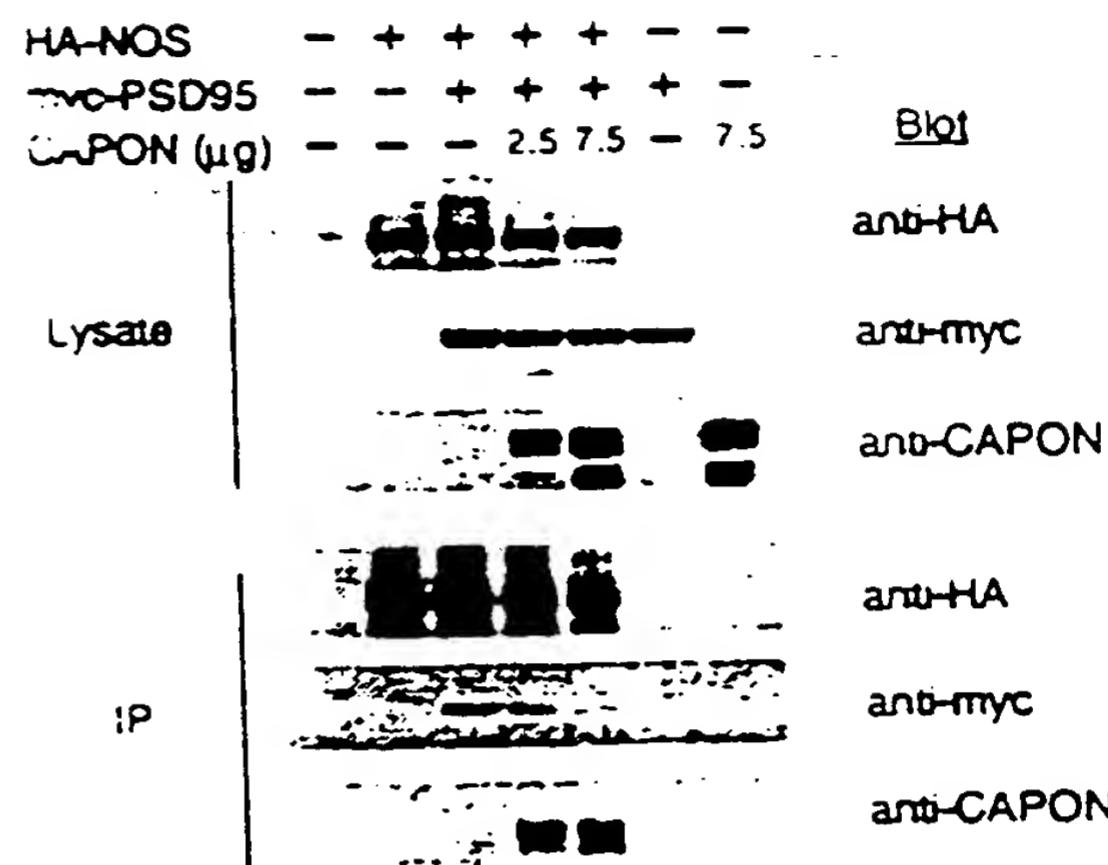


Figure 6. CAPON Expression Prevents the Interaction of PSD95 and nNOS

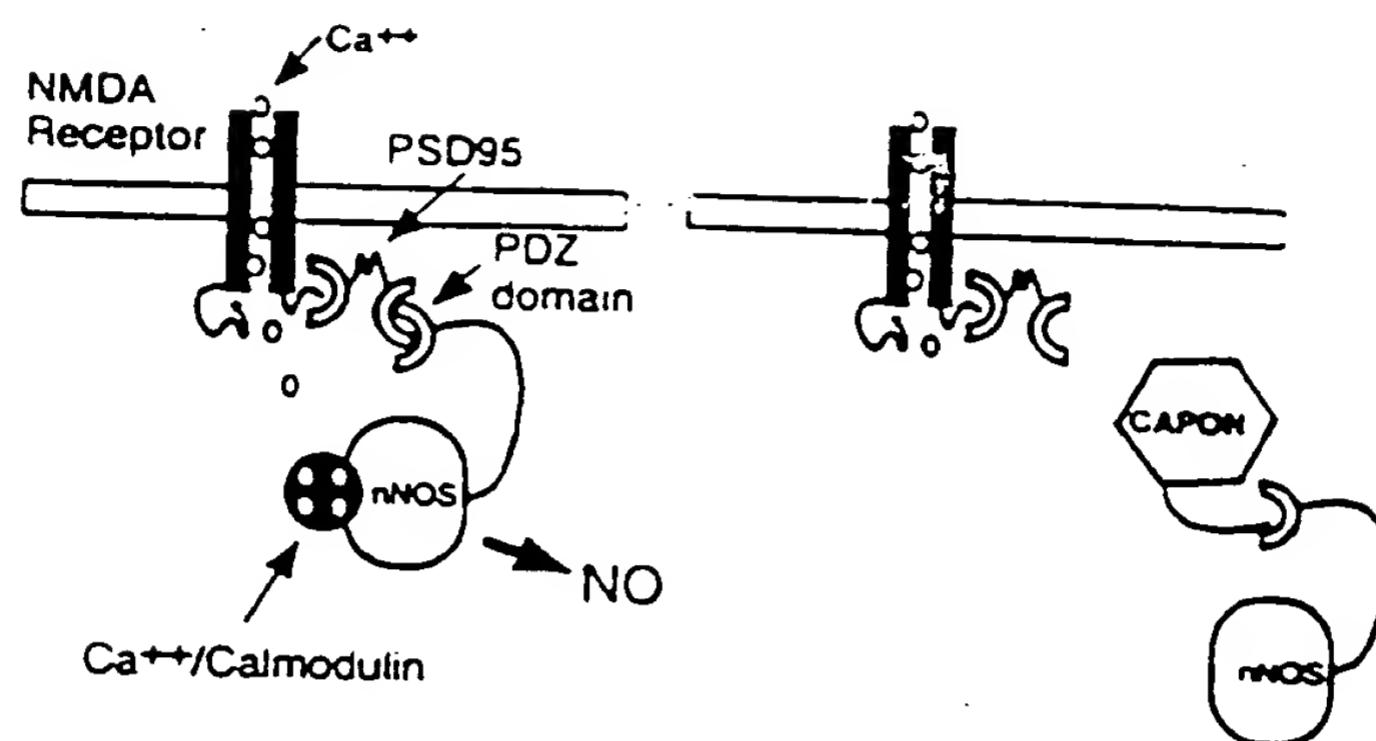


Figure 7. Model of PSD95/nNOS Regulation by CAPON

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

(i) APPLICANT: Snyder, Solomon  
Jaffrey, Samie

5 (ii) TITLE OF THE INVENTION: CAPON, a protein that binds  
neuronal nitric oxide synthase

(iii) NUMBER OF SEQUENCES: 6

## (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Banner & Witcoff  
(B) STREET: 1001 G Street, NW  
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(D) STATE: DC  
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(F) ZIP: 20001

15 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette  
(B) COMPUTER: IBM Compatible  
(C) OPERATING SYSTEM: DOS  
(D) SOFTWARE: FastSEQ for Windows Version 2.0

20 (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE: 22-JAN-1998  
(C) CLASSIFICATION:

25 (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:

30 (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Kagan, Sarah A  
(B) REGISTRATION NUMBER: 32141  
(C) REFERENCE/DOCKET NUMBER: 01107.73424

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 202-508-9100  
(B) TELEFAX: 202-508-9299  
(C) TELEX:

35 (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2826 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- 5 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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 120 CCGCCTGCCCG GGCCGCCTGG CCGCCTCCCC GTAGTCAGAG CGCGGCCACC GAGCTGCTCG  
 180  
 15 CGCCAGCCGC ATCCCGCGCCG CCCCTGCCGA TCGGCCCTCC GGAGGCACCG CTCCGGGTCC  
 240  
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 300  
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 480  
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 720  
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 840  
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 900  
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 1140  
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 1200  
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 1860 ACGAAAGCGA GGAGCGTGAC TCGTGGTCGC AGGAAGAGCT GCCACGCCTG CTCAATGTCC  
 20 1920 TACAGCGGCA GGAGTTGGGT GACAGTTGG ATGATGAGAT CGCCGTGTAG GTGCAGGGCA  
 1980 AGGAGCTGGT GAAGGTGGCA GCATGATGCC AAGGGGGTCA AGTCTGCCTG TCCCCGGCTG  
 2040 GGGAAAGCCA GGGGAAAGCA CCGCTGAGAA AAACACCCAG GGCTGAGAGT GTAGGGTTTC  
 25 2100 AGAAGAGGGT TGGGATTTG CTTTGGAAGG TAAAGCAGGG AAGAAAATGG ATTCTAGAC  
 2160 ACAGGAATCA GCACCTGTAT TCTGCTAATG ACTGAATGGG ACGGAAGCAG GGCTTTCCAG  
 2220 AACCCAGGAC CTTGGGATGG GTCCGCCTTC AAGAATCACA GTTCTGGAAG GCCTGTTGCT  
 30 2280 CCCACCGTTA TAGTCAGGTT CTACTCAATC TGTCCTGTAG GTCTCAGTGG CCTACACTCT  
 2340 CCTGTCTCTG TGGTGCAGAT CATAAATGGA AGCCATTGAT ACCGTCTCAC GTACTTTGTT  
 35 2400 TTGGATATCA GGATGCTACA AGTTGCCTAA CCCTCCCTTA AGCTGTAGGA GAATTCCCTC  
 2460 CCCAGGCCCT GGCTGAGATC AGAGAGGTTG GAGGATTTCC CTCACTGCTG GGAAATTGAG  
 40 2520 ACTCTGCCAT TCAGTGAGCA TGGAGGTGAC AGCAGTCACA AGTCACAGTG AATAAACTAG  
 2580 GAATTTACTC TAAGTGGGGT GTTGGATGTT GCTTCTGAGG AAGCTAGGAG TATGAATAGG  
 2640 ATTGAGGACC CTGCAGGGAG AGCCTGGGA GGGTTAGCCT AGGGGAGGGT TAGCCTAGAG  
 45 2700 AAGGGTTAGC CTAGGAGTGC TGATGACAGT TGTGGCAGCT CATGTAGGTG TGATTCTCA  
 2760 GTTTGGAAAC CATGCCCTT ACCCATCTCC TGCCTGCAAC CCAGCTCATA TAAACGAGGC  
 50 2820 TAAGAACTAT CATAATATCC CCTTTCTTG CCTCAGGGC TGTGCCTGCC TAATGAGTGC  
 2826 GGCCGC

(2) INFORMATION FOR SEQ ID NO:2:

5 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 503 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

	Met	Pro	Ser	Lys	Thr	Lys	Tyr	Asn	Leu	Val	Asp	Asp	Gly	His	Asp	Leu
10	1				5					10				15		
	Arg	Ile	Pro	Leu	His	Asn	Glu	Asp	Ala	Phe	Gln	His	Gly	Ile	Ser	Phe
					20					25				30		
	Glu	Ala	Lys	Tyr	Val	Gly	Ser	Leu	Asp	Val	Pro	Arg	Pro	Asn	Ser	Arg
					35					40				45		
15	Val	Glu	Ile	Val	Ala	Ala	Met	Arg	Arg	Ile	Arg	Tyr	Glu	Phe	Lys	Ala
	50				55					60						
	Lys	Asn	Ile	Lys	Lys	Lys	Val	Ser	Ile	Met	Val	Ser	Val	Asp	Gly	
	65				70					75				80		
	Val	Lys	Val	Ile	Leu	Lys	Lys	Lys	Lys	Lys	Lys	Glu	Trp	Thr	Trp	
20					85					90				95		
	Asp	Glu	Ser	Lys	Met	Leu	Val	Met	Gln	Asp	Pro	Ile	Tyr	Arg	Ile	Phe
					100				105				110			
	Tyr	Val	Ser	His	Asp	Ser	Gln	Asp	Leu	Lys	Ile	Phe	Ser	Tyr	Ile	Ala
					115				120				125			
25	Arg	Asp	Gly	Ala	Ser	Asn	Ile	Phe	Arg	Cys	Asn	Val	Phe	Lys	Ser	Lys
	130				135				140							
	Lys	Lys	Ser	Gln	Ala	Met	Arg	Ile	Val	Arg	Thr	Val	Gly	Gln	Ala	Phe
	145				150				155						160	
	Glu	Val	Cys	His	Lys	Leu	Ser	Leu	Gln	His	Thr	Gln	Gln	Asn	Ala	Asp
					165				170						175	
30	Gly	Gln	Glu	Asp	Gly	Glu	Ser	Glu	Arg	Asn	Ser	Asp	Gly	Ser	Gly	Asp
					180				185				190			
	Pro	Gly	Arg	Gln	Leu	Thr	Gly	Ala	Glu	Arg	Val	Ser	Thr	Ala	Thr	Ala
					195				200				205			
35	Glu	Glu	Thr	Asp	Ile	Asp	Ala	Val	Glu	Val	Pro	Leu	Pro	Gly	Asn	Asp
					210				215				220			
	Ile	Leu	Glu	Phe	Ser	Arg	Gly	Val	Thr	Asp	Leu	Asp	Ala	Ile	Gly	Lys
	225				230				235					240		
	Asp	Gly	Gly	Ser	His	Ile	Asp	Thr	Thr	Val	Ser	Pro	His	Pro	Gln	Glu
					245				250					255		
40	Pro	Met	Leu	Ala	Ala	Ser	Pro	Arg	Met	Leu	Leu	Pro	Ser	Ser	Ser	Ser
					260				265				270			
	Ser	Lys	Pro	Pro	Gly	Leu	Gly	Thr	Gly	Thr	Pro	Leu	Ser	Thr	His	His
					275				280				285			
45	Gln	Met	Gln	Leu	Leu	Gln	Gln	Leu	Leu	Gln	Gln	Gln	Gln	Gln	Gln	Thr
					290				295				300			
	Gln	Val	Ala	Val	Ala	Gln	Val	His	Leu	Leu	Lys	Asp	Gln	Leu	Ala	Ala
	305				310				315					320		
	Glu	Ala	Ala	Ala	Arg	Leu	Glu	Ala	Gln	Ala	Arg	Val	His	Gln	Leu	Leu
					325				330					335		
50	Leu	Gln	Asn	Lys	Asp	Met	Leu	Gln	His	Ile	Ser	Leu	Leu	Val	Lys	Gln
					340				345					350		

	Val	Gln	Glu	Leu	Glu	Leu	Lys	Leu	Ser	Gly	Gln	Ser	Thr	Met	Gly	Ser
	355									360				365		
	Gln	Asp	Ser	Leu	Leu	Glu	Ile	Thr	Phe	Arg	Ser	Gly	Ala	Leu	Pro	Val
	370									375				380		
5	Leu	Cys	Glu	Ser	Thr	Thr	Pro	Lys	Pro	Glu	Asp	Leu	His	Ser	Pro	Leu
	385									390			395			400
	Leu	Gly	Ala	Gly	Leu	Ala	Asp	Phe	Ala	His	Pro	Val	Gly	Ser	Pro	Leu
										405			410			415
10	Gly	Arg	Arg	Asp	Cys	Leu	Val	Lys	Leu	Glu	Cys	Phe	Arg	Phe	Leu	Pro
										420			425			430
	Ala	Glu	Asp	Asn	Gln	Pro	Met	Ala	Gln	Gly	Glu	Pro	Leu	Leu	Gly	Gly
										435			440			445
	Leu	Glu	Leu	Ile	Lys	Phe	Arg	Glu	Ser	Gly	Ile	Ala	Ser	Glu	Tyr	Glu
										450			455			460
15	Ser	Asn	Thr	Asp	Glu	Ser	Glu	Glu	Arg	Asp	Ser	Trp	Ser	Gln	Glu	Glu
										465			470			480
	Leu	Pro	Arg	Leu	Leu	Asn	Val	Leu	Gln	Arg	Gln	Glu	Leu	Gly	Asp	Ser
										485			490			495
20	Leu	Asp	Asp	Glu	Ile	Ala	Val									
										500						

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1504 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

	AAGCTTGGCA	CGAGGTCAAG	CAGGTGCAAG	AGCTGGA	ACT	GAAGCTGTCA	GGACAGAACG	
30	60	CCATGGGCTC	CCAGGACAGC	TTGCTGGAGA	TCACCTTCCG	CTCCGGAGCC	CTGCCCGTGC	
	120	TCTGTGACCC	CACGACCCCT	AAGCCAGAGG	ACCTGCATT	GCCGCCGCTG	GGCGCGGGCT	
	180	TGGCTGACTT	TGCCCA	CGGGCAGCC	CCTTAGGTAG	GCGCGACTGC	TTGGTGAAGC	
35	240	TGGAGTGCTT	TCGCTTTCTT	CCGCCCGAGG	ACACCCCGCC	CCCAGCGCAG	GGCGAGGCAG	
	300	TCC	GGGCAGGCTC	ATCAAGTTCC	GAGAGTCAGG	CATGCCCTCG	GAGTACGAGT	
40	360	CCAACACCGA	CGAGAGCGAG	GAGCGCGACT	CGTGGTCCCA	GGAGGAGCTG	CCGCGCCTGC	
	420	TGAATGT	CCT	GCAGAGGCAG	GAAC	TGGAGCTGGAG	TGATGAGATC	GCCGTGTAGG
45	480	TGCCGAGGGC	GAGGAGATGG	AGGC	GGCGCGGC	GTGGCTGGAG	GGGCCGTGTC	TGGCTGCTGC
	540	CGGGTAGGG	GATGCCAGT	GAATGTGCAC	TGCCGAGGAG	AATGCCAGCC	AGGGCCCGGG	
	600							

660 AGAGTGTGAG GTTCAGGAA AGTATTGAGA TTCTGCTTG GAGGGTAAAG TGGGAAAGAA  
 660  
 660 ATCGGATTCC CAGAGGTGAA TCAGCTCCTC TCCTACTTGT GACTAGAGGG TGGTGGAGGT  
 720  
 720  
 5 AAGGCCTTCC AGAGCCCAG GCTTCAGGAG AGGGTCTCTC TCCAGGACTG CCAGGCTGCT  
 780  
 780  
 840 GGAGGACCTG CCCCTACCTG CTGCATCGTC AGGCTCCCAC GCTTGTCGG TGATGCC  
 840  
 840  
 10 CTACCCCCCTC ACTCTCCCCG TCTCCATGGT CCCGACCAGG AAGGGAAGCC ATCGGTACCT  
 900  
 900  
 960 TCTCAGGTAC TTTGTTCTG GATATCACGA TGCTGCGAGT TGCTTAACCC TCCCCCTACC  
 960  
 960  
 1020 TTTATGAGAG GAATTCCCTC TCCAGGCCCT TGCTGAGATT GTAGAGATTG AGTGCTCTGG  
 1020  
 1020  
 15 ACCGCAGAAAG CCAGGCTAGT CCTTGAGGG TGAGCATGGA ATTGGAATGT GTCACAGTGG  
 1080  
 1080  
 1140 ATAAGCTTTT AGAGGAACAG AATCCAAACA TTTCTCCAG CCGGACATTG AATGTTGCTA  
 1140  
 1140  
 20 CAAAGGGAGC CTTGAAGCTT TAACATGGTT CAGGCCCTG GTGTGAGAGC CCAGGGGGAG  
 1200  
 1200  
 1260 GACAGCTTGT CTGCTGCTCC AAATCACTTA GATCTGATTG CTGTTTGAA AGTCCTGCC  
 1260  
 1260  
 1320 TGCCTTCCTC CTGCCTGTAG CCCAGCCAT CAAATGAA GCTGGAAATT GCCCCTCACC  
 1320  
 1320  
 1380 25 TCCCCTGTGT CCTGTCCAGC TGAAGCTTT GCAGCACTT ACCTCTCTGA AAGCCCCAGA  
 1380  
 1380  
 1440 GGACCAAGAGC CCCCAGCCTT ACCTCTCAAC CTGTCCCTC CACTGGCAG TGGTGGTCAG  
 1440  
 1440  
 30 1500 TTTTACTGC AAAAAAAA AAAAAGAAAA AAGAGAAAA AAAAAAAA ATTCCCTGCGG  
 1500  
 1500  
 1504 CCGC  
 1504

## (2) INFORMATION FOR SEQ ID NO:4:

35 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 153 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Lys Gln Val Gln Glu Leu Glu Leu Lys Leu Ser Gly Gln Asn Ala Met  
 1 5 10 15  
 Gly Ser Gln Asp Ser Leu Leu Glu Ile Thr Phe Arg Ser Gly Ala Leu  
 20 25 30  
 45 Pro Val Leu Cys Asp Pro Thr Thr Pro Lys Pro Glu Asp Leu His Ser  
 35 40 45  
 Pro Pro Leu Gly Ala Gly Leu Ala Asp Phe Ala His Pro Ala Gly Ser  
 50 55 60  
 Pro Leu Gly Arg Arg Asp Cys Leu Val Lys Leu Glu Cys Phe Arg Phe

5	65	70	75	80
	Leu Pro Pro Glu Asp Thr Pro Pro Pro Ala Gln Gly Glu Ala Leu Leu			
		85	90	95
	Gly Gly Leu Glu Leu Ile Lys Phe Arg Glu Ser Gly Ile Ala Ser Glu			
		100	105	110
	Tyr Glu Ser Asn Thr Asp Glu Ser Glu Glu Arg Asp Ser Trp Ser Gln			
		115	120	125
	Glu Glu Leu Pro Arg Leu Leu Asn Val Leu Gln Arg Gln Glu Leu Gly			
		130	135	140
10	Asp Gly Leu Asp Asp Glu Ile Ala Val			
		145	150	

(2) INFORMATION FOR SEQ ID NO:5:

**(i) SEQUENCE CHARACTERISTICS:**

- (A) LENGTH: 1430 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

20	Met	Glu	Glu	Asn	Thr	Phe	Gly	Val	Gln	Gln	Ile	Gln	Pro	Asn	Val	Ile
	1				5				10						15	
	Ser	Val	Arg	Leu	Phe	Lys	Arg	Lys	Val	Gly	Gly	Leu	Gly	Phe	Leu	Val
				20					25					30		
25	Lys	Glu	Arg	Val	Ser	Lys	Pro	Pro	Val	Ile	Ile	Ser	Asp	Leu	Ile	Arg
				35					40				45			
	Gly	Gly	Ala	Ala	Glu	Gln	Ser	Gly	Leu	Ile	Gln	Ala	Gly	Asp	Ile	Ile
				50					55			60				
30	Leu	Ala	Val	Asn	Asp	Arg	Pro	Leu	Val	Asp	Leu	Ser	Tyr	Asp	Ser	Ala
				65				70			75			80		
	Leu	Glu	Val	Leu	Arg	Gly	Ile	Ala	Ser	Glu	Thr	His	Val	Val	Leu	Ile
					85					90				95		
	Leu	Arg	Gly	Pro	Glu	Gly	Phe	Thr	Thr	His	Leu	Glu	Thr	Thr	Phe	Thr
					100					105				110		
35	Gly	Asp	Gly	Thr	Pro	Lys	Thr	Ile	Arg	Val	Thr	Gln	Pro	Leu	Gly	Pro
					115					120				125		
	Pro	Thr	Lys	Ala	Val	Asp	Leu	Ser	His	Gln	Pro	Ser	Ala	Ser	Lys	Asp
				130					135				140			
	Gln	Ser	Leu	Ala	Val	Asp	Arg	Val	Thr	Gly	Leu	Gly	Asn	Gly	Pro	Gln
				145				150			155			160		
40	His	Ala	Gln	Gly	His	Gly	Gln	Gly	Ala	Gly	Ser	Val	Ser	Gln	Ala	Asn
					165					170				175		
	Gly	Val	Ala	Ile	Asp	Pro	Thr	Met	Lys	Ser	Thr	Lys	Ala	Asn	Leu	Gln
					180					185				190		
45	Asp	Ile	Gly	Glu	His	Asp	Glu	Leu	Leu	Lys	Glu	Ile	Glu	Pro	Val	Leu
					195					200				205		
	Ser	Ile	Leu	Asn	Ser	Gly	Ser	Lys	Ala	Thr	Asn	Arg	Gly	Gly	Pro	Ala
				210					215				220			
	Lys	Ala	Glu	Met	Lys	Asp	Thr	Gly	Ile	Gln	Val	Asp	Arg	Asp	Leu	Asp
				225				230				235				240

	Gly Lys Ser His Lys Ala Pro Pro Leu Gly Gly Asp Asn Asp Arg Val			
	245	250	255	
	Phe Asn Asp Leu Trp Gly Lys Asp Asn Val Pro Val Ile Leu Asn Asn			
	260	265	270	
5	Pro Tyr Ser Glu Lys Glu Gln Ser Pro Thr Ser Gly Lys Gln Ser Pro			
	275	280	285	
	Thr Lys Asn Gly Ser Pro Ser Arg Cys Pro Arg Phe Leu Lys Val Lys			
	290	295	300	
10	Asn Trp Glu Thr Asp Val Val Leu Thr Asp Thr Leu His Leu Lys Ser			
	305	310	315	320
	Thr Leu Glu Thr Gly Cys Thr Glu His Ile Cys Met Gly Ser Ile Met			
	325	330	335	
	Leu Pro Ser Gln His Thr Arg Lys Pro Glu Asp Val Arg Thr Lys Asp			
	340	345	350	
15	Gln Leu Phe Pro Leu Ala Lys Glu Phe Leu Asp Gln Tyr Tyr Ser Ser			
	355	360	365	
	Ile Lys Arg Phe Gly Ser Lys Ala His Met Asp Arg Leu Glu Glu Val			
	370	375	380	
20	Asn Lys Glu Ile Glu Ser Thr Ser Thr Tyr Gln Leu Lys Asp Thr Glu			
	385	390	395	400
	Leu Ile Tyr Gly Ala Lys His Ala Trp Arg Asn Ala Ser Arg Cys Val			
	405	410	415	
	Gly Arg Ile Gln Trp Ser Lys Leu Gln Val Phe Asp Ala Arg Asp Cys			
	420	425	430	
25	Thr Thr Ala His Gly Met Phe Asn Tyr Ile Cys Asn His Val Lys Tyr			
	435	440	445	
	Ala Thr Asn Lys Gly Asn Leu Arg Ser Ala Ile Thr Ile Phe Pro Gln			
	450	455	460	
	Arg Thr Asp Gly Lys His Asp Phe Arg Val Trp Asn Ser Gln Leu Ile			
30	465	470	475	480
	Arg Tyr Ala Gly Tyr Lys Gln Pro Asp Gly Ser Thr Leu Gly Asp Pro			
	485	490	495	
	Ala Asn Val Gln Phe Thr Glu Ile Cys Ile Gln Gln Gly Trp Lys Ala			
	500	505	510	
35	Pro Arg Gly Arg Phe Asp Val Leu Pro Leu Leu Leu Gln Ala Asn Gly			
	515	520	525	
	Asn Asp Pro Glu Leu Phe Gln Ile Pro Pro Glu Leu Val Leu Glu Val			
	530	535	540	
40	Pro Ile Arg His Pro Lys Phe Asp Trp Phe Lys Asp Leu Gly Leu Lys			
	545	550	555	560
	Trp Tyr Gly Leu Pro Ala Val Ser Asn Met Leu Leu Glu Ile Gly Gly			
	565	570	575	
	Leu Glu Phe Ser Ala Cys Pro Phe Ser Gly Trp Tyr Met Gly Thr Glu			
	580	585	590	
45	Ile Gly Val Arg Asp Tyr Cys Asp Asn Ser Arg Tyr Asn Ile Leu Glu			
	595	600	605	
	Glu Val Ala Lys Lys Met Asp Leu Asp Met Arg Lys Thr Ser Ser Leu			
	610	615	620	
50	Trp Lys Asp Gln Ala Leu Val Glu Ile Asn Ile Ala Val Leu Tyr Ser			
	625	630	635	640
	Phe Gln Ser Asp Lys Val Thr Ile Val Asp His His Ser Ala Thr Glu			
	645	650	655	
	Ser Phe Ile Lys His Met Glu Asn Glu Tyr Arg Cys Arg Gly Gly Cys			
	660	665	670	

	Pro Ala Asp Trp Val Trp Ile Val Pro Pro Met Ser Gly Ser Ile Thr		
	675	680	685
	Pro Val Phe His Gln Glu Met Leu Asn Tyr Arg Leu Thr Pro Ser Phe		
	690	695	700
5	Glu Tyr Gln Pro Asp Pro Trp Asn Thr His Val Trp Lys Gly Thr Asn		
	705	710	715
	Gly Thr Pro Thr Lys Arg Arg Ala Ile Gly Phe Lys Lys Leu Ala Glu		
	725	730	735
10	Ala Val Lys Phe Ser Ala Lys Leu Met Gly Gln Ala Met Ala Lys Arg		
	740	745	750
	Val Lys Ala Thr Ile Leu Tyr Ala Thr Glu Thr Gly Lys Ser Gln Ala		
	755	760	765
	Tyr Ala Lys Thr Leu Cys Glu Ile Phe Lys His Ala Phe Asp Ala Lys		
	770	775	780
15	Ala Met Ser Met Glu Glu Tyr Asp Ile Val His Leu Glu His Glu Ala		
	785	790	795
	Leu Val Leu Val Val Thr Ser Thr Phe Gly Asn Gly Asp Pro Pro Glu		
	805	810	815
20	Asn Gly Glu Lys Phe Gly Cys Ala Leu Met Glu Met Arg His Pro Asn		
	820	825	830
	Ser Val Gln Glu Glu Arg Lys Ser Tyr Lys Val Arg Phe Asn Ser Val		
	835	840	845
	Ser Ser Tyr Ser Asp Ser Arg Lys Ser Ser Gly Asp Gly Pro Asp Leu		
	850	855	860
25	Arg Asp Asn Phe Glu Ser Thr Gly Pro Leu Ala Asn Val Arg Phe Ser		
	865	870	875
	Val Phe Gly Leu Gly Ser Arg Ala Tyr Pro His Phe Cys Ala Phe Gly		
	885	890	895
30	His Ala Val Asp Thr Leu Leu Glu Glu Leu Gly Gly Glu Arg Ile Leu		
	900	905	910
	Lys Met Arg Glu Gly Asp Glu Leu Cys Gly Gln Glu Glu Ala Phe Arg		
	915	920	925
	Thr Trp Ala Lys Lys Val Phe Lys Ala Ala Cys Asp Val Phe Cys Val		
	930	935	940
35	Gly Asp Asp Val Asn Ile Glu Lys Pro Asn Asn Ser Leu Ile Ser Asn		
	945	950	955
	Asp Arg Ser Trp Lys Arg Asn Lys Phe Arg Leu Thr Tyr Val Ala Glu		
	965	970	975
40	Ala Pro Asp Leu Thr Gln Gly Leu Ser Asn Val His Lys Lys Arg Val		
	980	985	990
	Ser Ala Ala Arg Leu Leu Ser Arg Gln Asn Leu Gln Ser Pro Lys Phe		
	995	1000	1005
	Ser Arg Ser Thr Ile Phe Val Arg Leu His Thr Asn Gly Asn Gln Glu		
	1010	1015	1020
45	Leu Gln Tyr Gln Pro Gly Asp His Leu Gly Val Phe Pro Gly Asn His		
	1025	1030	1035
	Glu Asp Leu Val Asn Ala Leu Ile Glu Arg Leu Glu Asp Ala Pro Pro		
	1045	1050	1055
50	Ala Asn His Val Val Lys Val Glu Met Leu Glu Glu Arg Asn Thr Ala		
	1060	1065	1070
	Leu Gly Val Ile Ser Asn Trp Lys Asp Glu Ser Arg Leu Pro Pro Cys		
	1075	1080	1085
	Thr Ile Phe Gln Ala Phe Lys Tyr Tyr Leu Asp Ile Thr Thr Pro Pro		
	1090	1095	1100

Thr Pro Leu Gln Leu Gln Gln Phe Ala Ser Leu Ala Thr Asn Glu Lys  
 105 1110 1115 1120  
 Glu Lys Gln Arg Leu Leu Val Leu Ser Lys Gly Leu Gln Glu Tyr Glu  
 1125 1130 1135  
 5 Glu Trp Lys Trp Gly Lys Asn Pro Thr Met Val Glu Val Leu Glu Glu  
 1140 1145 1150  
 Phe Pro Ser Ile Gln Met Pro Ala Thr Leu Leu Leu Thr Gln Leu Ser  
 1155 1160 1165  
 10 Leu Leu Gln Pro Arg Tyr Tyr Ser Ile Ser Ser Pro Asp Met Tyr  
 1170 1175 1180  
 Pro Asp Glu Val His Leu Thr Val Ala Ile Val Ser Tyr His Thr Arg  
 1185 1190 1195 1200  
 Asp Gly Glu Gly Pro Val His His Gly Val Cys Ser Ser Trp Leu Asn  
 1205 1210 1215  
 15 Arg Ile Gln Ala Asp Asp Val Val Pro Cys Phe Val Arg Gly Ala Pro  
 1220 1225 1230  
 Ser Phe His Leu Pro Arg Asn Pro Gln Val Pro Cys Ile Leu Val Gly  
 1235 1240 1245  
 20 Pro Gly Thr Gly Ile Ala Pro Phe Arg Ser Phe Trp Gln Gln Arg Gln  
 1250 1255 1260  
 Phe Asp Ile Gln His Lys Gly Met Asn Pro Cys Pro Met Val Leu Val  
 1265 1270 1275 1280  
 Phe Gly Cys Arg Gln Ser Lys Ile Asp His Ile Tyr Arg Glu Glu Thr  
 1285 1290 1295  
 25 Leu Gln Ala Lys Asn Lys Gly Val Phe Arg Glu Leu Tyr Thr Ala Tyr  
 1300 1305 1310  
 Ser Arg Glu Pro Asp Arg Pro Lys Lys Tyr Val Gln Asp Val Leu Gln  
 1315 1320 1325  
 30 Glu Gln Leu Ala Glu Ser Val Tyr Arg Ala Leu Lys Glu Gln Gly Gly  
 1330 1335 1340  
 His Ile Tyr Val Cys Gly Asp Val Thr Met Ala Ala Asp Val Leu Lys  
 1345 1350 1355 1360  
 Ala Ile Gln Arg Ile Met Thr Gln Gln Gly Lys Leu Ser Glu Glu Asp  
 1365 1370 1375  
 35 Ala Gly Val Phe Ile Ser Arg Leu Arg Asp Asp Asn Arg Tyr His Glu  
 1380 1385 1390  
 Asp Ile Phe Gly Val Thr Leu Arg Thr Tyr Glu Val Thr Asn Arg Leu  
 1395 1400 1405  
 40 Arg Ser Glu Ser Ile Ala Phe Ile Glu Glu Ser Lys Lys Asp Ala Asp  
 1410 1415 1420  
 Glu Val Phe Ser Ser Pro  
 425 1430

## (2) INFORMATION FOR SEQ ID NO:6:

45 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1554 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Glu Asp His Met Phe Gly Val Gln Gln Ile Gln Pro Asn Val Ile  
 1 5 10 15  
 Ser Val Arg Leu Phe Lys Arg Lys Val Gly Gly Leu Gly Phe Leu Val  
 20 25 30  
 5 Lys Glu Arg Val Ser Lys Pro Pro Val Ile Ile Ser Asp Leu Ile Arg  
 35 40 45  
 Gly Gly Ala Ala Glu Gln Ser Gly Leu Ile Gln Ala Gly Asp Ile Ile  
 50 55 60  
 Leu Ala Val Asn Gly Arg Pro Leu Val Asp Leu Ser Tyr Asp Ser Ala  
 10 65 70 75 80  
 Leu Glu Val Leu Arg Gly Ile Ala Ser Glu Thr His Val Val Leu Ile  
 85 90 95  
 Leu Arg Gly Pro Glu Gly Phe Thr Thr His Leu Glu Thr Thr Phe Thr  
 100 105 110  
 15 Gly Asp Gly Thr Pro Lys Thr Ile Arg Val Thr Gln Pro Leu Gly Pro  
 115 120 125  
 Pro Thr Lys Ala Val Asp Leu Ser His Gln Pro Pro Ala Gly Lys Glu  
 130 135 140  
 Gln Pro Leu Ala Val Asp Gly Ala Ser Gly Pro Gly Asn Gly Pro Gln  
 20 145 150 155 160  
 His Ala Tyr Asp Asp Gly Gln Glu Ala Gly Ser Leu Pro His Ala Asn  
 165 170 175  
 Gly Leu Ala Pro Arg Pro Pro Gly Gln Asp Pro Ala Lys Lys Ala Thr  
 180 185 190  
 25 Arg Val Ser Leu Gln Gly Arg Gly Glu Asn Asn Glu Leu Leu Lys Glu  
 195 200 205  
 Ile Glu Pro Val Leu Ser Leu Leu Thr Ser Gly Ser Arg Gly Val Lys  
 210 215 220  
 Gly Gly Ala Pro Ala Lys Ala Glu Met Lys Asp Met Gly Ile Gln Val  
 30 225 230 235 240  
 Asp Arg Asp Leu Asp Gly Lys Ser His Lys Pro Leu Pro Leu Gly Val  
 245 250 255  
 Glu Asn Asp Arg Val Phe Asn Asp Leu Trp Gly Lys Gly Asn Val Pro  
 260 265 270  
 35 Val Val Leu Asn Asn Pro Tyr Ser Glu Lys Glu Gln Pro Pro Thr Ser  
 275 280 285  
 Gly Lys Gln Ser Pro Thr Lys Asn Gly Ser Pro Ser Lys Cys Pro Arg  
 290 295 300  
 Phe Leu Lys Val Lys Asn Trp Glu Thr Glu Val Val Leu Thr Asp Thr  
 40 305 310 315 320  
 Leu His Leu Lys Ser Thr Leu Glu Thr Gly Cys Thr Glu Tyr Ile Cys  
 325 330 335  
 Met Gly Ser Ile Met His Pro Ser Gln His Ala Arg Arg Pro Glu Asp  
 340 345 350  
 45 Val Arg Thr Lys Gly Gln Leu Phe Pro Leu Ala Lys Glu Phe Ile Asp  
 355 360 365  
 Gln Tyr Tyr Ser Ser Ile Lys Arg Phe Gly Ser Lys Ala His Met Glu  
 370 375 380  
 Arg Leu Glu Glu Val Asn Lys Glu Ile Asp Thr Thr Ser Thr Tyr Gln  
 50 385 390 395 400  
 Leu Lys Asp Thr Glu Leu Ile Tyr Gly Ala Lys His Ala Trp Arg Asn  
 405 410 415  
 Ala Ser Arg Cys Val Gly Arg Ile Gln Trp Ser Lys Leu Gln Val Phe  
 420 425 430

	Asp Ala Arg Asp Cys Thr Thr Ala His Gly Met Phe Asn Tyr Ile Cys	
	435 440 445	
	Asn His Val Lys Tyr Ala Thr Asn Lys Gly Asn Leu Arg Ser Ala Ile	
	450 455 460	
5	Thr Ile Phe Pro Gln Arg Thr Asp Gly Lys His Asp Phe Arg Val Trp	
	465 470 475 480	
	Asn Ser Gln Leu Ile Arg Tyr Ala Gly Tyr Lys Gln Pro Asp Gly Ser	
	485 490 495	
10	Thr Leu Gly Asp Pro Ala Asn Val Gln Phe Thr Glu Ile Cys Ile Gln	
	500 505 510	
	Gln Gly Trp Lys Pro Pro Arg Gly Arg Phe Asp Val Leu Pro Leu Leu	
	515 520 525	
	Leu Gln Ala Asn Gly Asn Asp Pro Glu Leu Phe Gln Ile Pro Pro Glu	
	530 535 540	
15	Leu Val Leu Glu Val Pro Ile Arg His Pro Lys Phe Glu Trp Phe Lys	
	545 550 555 560	
	Asp Leu Gly Leu Lys Trp Tyr Gly Leu Pro Ala Val Ser Asn Met Leu	
	565 570 575	
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	580 585 590	
	Tyr Met Gly Thr Glu Ile Gly Val Arg Asp Tyr Cys Asp Asn Ser Arg	
	595 600 605	
	Tyr Asn Ile Leu Glu Glu Val Ala Lys Lys Met Asn Leu Asp Met Arg	
	610 615 620	
25	Lys Thr Ser Ser Leu Trp Lys Asp Gln Ala Leu Val Glu Ile Asn Ile	
	625 630 635 640	
	Ala Val Leu Tyr Ser Phe Gln Ser Asp Lys Val Thr Ile Val Asp His	
	645 650 655	
30	His Ser Ala Thr Glu Ser Phe Ile Lys His Met Glu Asn Glu Tyr Arg	
	660 665 670	
	Cys Arg Gly Gly Cys Pro Ala Asp Trp Val Trp Ile Val Pro Pro Met	
	675 680 685	
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	690 695 700	
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	Trp Lys Gly Thr Asn Gly Thr Pro Thr Lys Arg Arg Ala Ile Gly Phe	
	725 730 735	
40	Lys Lys Leu Ala Glu Ala Val Lys Phe Ser Ala Lys Leu Met Gly Gln	
	740 745 750	
	Ala Met Ala Lys Arg Val Lys Ala Thr Ile Leu Tyr Ala Thr Glu Thr	
	755 760 765	
	Gly Lys Ser Gln Ala Tyr Ala Lys Thr Leu Cys Glu Ile Phe Lys His	
	770 775 780	
45	Ala Phe Asp Ala Lys Val Met Ser Met Glu Glu Tyr Asp Ile Val His	
	785 790 795 800	
	Leu Glu His Glu Thr Leu Val Leu Val Val Thr Ser Thr Phe Gly Asn	
	805 810 815	
50	Gly Asp Pro Pro Glu Asn Gly Glu Lys Phe Gly Cys Ala Leu Met Glu	
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	835 840 845	
	Arg Phe Asn Ser Val Ser Ser Tyr Ser Asp Ser Gln Lys Ser Ser Gly	
	850 855 860	

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 Asn Val Arg Phe Ser Val Phe Gly Leu Gly Ser Arg Ala Tyr Pro His  
 885 890 895  
 5 Phe Cys Ala Phe Gly His Ala Val Asp Thr Leu Leu Glu Glu Leu Gly  
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 915 920 925  
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 930 935 940  
 10 Asp Val Phe Cys Val Gly Asp Asp Val Asn Ile Glu Lys Ala Asn Asn  
 945 950 955 960  
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 15 Thr Phe Val Ala Glu Ala Pro Glu Leu Thr Gln Gly Leu Ser Asn Val  
 980 985 990  
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 995 1000 1005  
 Gln Ser Pro Lys Ser Ser Arg Ser Thr Ile Phe Val Arg Leu His Thr  
 20 1010 1015 1020  
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 1025 1030 1035 1040  
 Phe Pro Gly Asn His Glu Asp Leu Val Asn Ala Leu Ile Glu Arg Leu  
 1045 1050 1055  
 25 Glu Asp Ala Pro Pro Val Asn Gln Met Val Lys Val Glu Leu Leu Glu  
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 Arg Leu Pro Pro Cys Thr Ile Phe Gln Ala Phe Lys Tyr Tyr Leu Asp  
 30 1090 1095 1100  
 Ile Thr Thr Pro Pro Thr Pro Leu Gln Leu Gln Phe Ala Ser Leu  
 105 1110 1115 1120  
 Ala Thr Ser Glu Lys Glu Lys Gln Arg Leu Leu Val Leu Ser Lys Gly  
 1125 1130 1135  
 35 Leu Gln Glu Tyr Glu Glu Trp Lys Trp Gly Lys Asn Pro Thr Ile Val  
 1140 1145 1150  
 Glu Val Leu Glu Glu Phe Pro Ser Ile Gln Met Pro Ala Thr Leu Leu  
 1155 1160 1165  
 Leu Thr Gln Leu Ser Leu Leu Gln Pro Arg Tyr Tyr Ser Ile Ser Ser  
 40 1170 1175 1180  
 Ser Pro Asp Met Tyr Pro Asp Glu Val His Leu Thr Val Ala Ile Val  
 185 1190 1195 1200  
 Ser Tyr Arg Thr Arg Asp Gly Glu Gly Pro Ile His His Gly Val Cys  
 1205 1210 1215  
 45 Ser Ser Trp Leu Asn Arg Ile Gln Ala Asp Glu Leu Val Pro Cys Phe  
 1220 1225 1230  
 Val Arg Gly Ala Pro Ser Phe His Leu Pro Arg Asn Pro Gln Val Pro  
 1235 1240 1245  
 Cys Ile Leu Val Gly Pro Gly Thr Gly Ile Ala Pro Phe Arg Ser Phe  
 1250 1255 1260  
 50 Trp Gln Gln Arg Gln Phe Asp Ile Gln His Lys Gly Met Asn Pro Cys  
 265 1270 1275 1280  
 Pro Met Val Leu Val Phe Gly Cys Arg Gln Ser Lys Ile Asp His Ile  
 1285 1290 1295

Tyr Arg Glu Glu Thr Leu Gln Ala Lys Asn Lys Gly Val Phe Arg Glu  
 1300 1305 1310  
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 1315 1320 1325  
 5 Gln Asp Ile Leu Gln Glu Gln Leu Ala Glu Ser Val Tyr Arg Ala Leu  
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 Lys Glu Gln Gly Gly His Ile Tyr Val Cys Gly Asp Val Thr Met Ala  
 345 1350 1355 1360  
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 10 1365 1370 1375  
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 1380 1385 1390  
 Asn Arg Tyr His Glu Asp Ile Phe Gly Val Thr Leu Arg Thr Tyr Glu  
 1395 1400 1405  
 15 Val Thr Asn Arg Leu Arg Ser Glu Ser Ile Ala Phe Ile Glu Glu Ser  
 1410 1415 1420  
 Lys Lys Asp Thr Asp Glu Gly Phe Gln Leu Leu Thr Gly Pro Ser Cys  
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 Pro Ala Gly Cys Lys Phe Cys Lys Arg Gly Gln Thr Leu Leu Asn Leu  
 20 1445 1450 1455  
 Ser Ser Gly Thr Pro Cys Gly Pro Arg Ser Ala Ser Cys Pro Cys Arg  
 1460 1465 1470  
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 1475 1480 1485  
 25 Arg Pro Ser Trp Val Tyr Ser Leu Ser Phe Pro Ala Ala Met Gln Cys  
 1490 1495 1500  
 Phe Ser Asn Leu Gln Trp Leu Leu Gln Asn Ser Val Pro Thr Pro Ser  
 505 1510 1515 1520  
 Leu Ala Asp Lys Gly Asn Ser Arg Val His Glu Thr Thr Gly Thr Trp  
 30 1525 1530 1535  
 Pro Ser Leu Trp Gly Phe Phe Ser Leu Gly Phe Pro Trp Lys Gly Cys  
 1540 1545 1550  
 Arg Asn

35 (2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 40 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GACTAGTCGA CTGAAGAGAA CACGTTGGG

30

(2) INFORMATION FOR SEQ ID NO:8:

45 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs  
 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

5           TCTGCAGATC TCAGTGGGCC TTGGAGCCAA A  
31

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/01199

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/47 G01N33/68 C12Q1/68 C12N15/62  
C07K16/18

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07K C12N G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JAFFREY S R ET AL: "PIN: AN ASSOCIATED PROTEIN INHIBITOR OF NSURONAL NITRIC OXIDE SYNTHASE" SCIENCE, vol. 274, 1 November 1996, pages 774-777, XP002050141 cited in the application see the whole document ---	
A	BRENMAN J E ET AL.: "Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alphal-syntrophin mediated by PDZ domains" CELL, vol. 84, 8 March 1996, pages 757-767, XP002104701 cited in the application see the whole document ---	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search

3 June 1999

Date of mailing of the international search report

16/06/1999

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Authorized officer

Oderwald, H

# INTERNATIONAL SEARCH REPORT

Interr. Application No.  
PCT/US 99/01199

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>JAFFREY S R ET AL.: "CAPON: a protein associated with neuronal nitric oxide synthase that regulates its interaction with PSD95"          NEURON,          vol. 20, no. 1, 23 January 1998, pages          115-124, XP002104610          see the whole document          -&amp; "accession number AF037070"          EMBL SEQUENCE DATABASE, 20 February 1998,          XP002104702          Heidelberg, Germany          see the whole document          -&amp; "accession number AF037071"          EMBL SEQUENCE DATABASE, 20 February 1998,          XP002104703          Heidelberg, Germany          see the whole document</p> <p>-----</p>	1-39

## INTERNATIONAL SEARCH REPORT

Int. application No.

PCT/US 99/01199

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  
**Remark:** Although claims 25-27 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/12, C07K 14/47, G01N 33/68, C12Q 1/68, C12N 15/62, C07K 16/18</b>		A1	(11) International Publication Number: <b>WO 99/37768</b>
			(43) International Publication Date: <b>29 July 1999 (29.07.99)</b>
(21) International Application Number: <b>PCT/US99/01199</b>		(74) Agents: <b>KAGAN, Sarah, A. et al.; Banner &amp; Witcoff, Ltd., 11th floor, 1001 G Street, N.W., Washington, DC 20001-4597 (US).</b>	
(22) International Filing Date: <b>21 January 1999 (21.01.99)</b>		(81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</b>	
(30) Priority Data: <b>09/010,998 22 January 1998 (22.01.98) US</b>		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application <b>US 09/010,998 (CON) Filed on 22 January 1998 (22.01.98)</b>			
(71) Applicant (for all designated States except US): <b>THE JOHNS HOPKINS UNIVERSITY [US/US]; 720 Rutland Avenue, Baltimore, MD 21205 (US).</b>			
(72) Inventors; and (75) Inventors/Applicants (for US only): <b>SNYDER, Solomon, H. [US/US]; Apartment 1001, 3801 Canterbury Road, Baltimore, MD 21218 (US). JAFFREY, Samie, R. [US/US]; Apartment 10B4 Reed, 1620 McElderry Street, Baltimore, MD 21205 (US).</b>			

(54) Title: CAPON: A PROTEIN ASSOCIATED WITH NEURONAL NITRIC OXIDE SYNTHASE

## (57) Abstract

Nitric oxide (NO) produced by neuronal nitric oxide synthase (nNOS) is important for N-methyl-D-aspartate (NMDA) receptor-dependent neurotransmitter release, neurotoxicity, and cyclic-GMP elevations. The coupling of NMDA receptor-mediated calcium influx and nNOS activation is postulated to be due to a physical coupling of the receptor and the enzyme by an intermediary adaptor protein PSD95, through a unique PDZ-PDZ domain interaction between PSD95 and nNOS. Here we report the identification of a novel nNOS-associated protein, CAPON, which is highly enriched in brain and has numerous colocalizations with nNOS. CAPON interacts with the nNOS PDZ domain through its C-terminus. CAPON competes with PSD95 for interaction with nNOS, and overexpression of CAPON results in a loss of PSD95/nNOS complexes in transfected cells. CAPON influences nNOS by regulating its ability to associate with PSD95/NMDA receptor complexes.

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## **CAPON: A PROTEIN ASSOCIATED WITH NEURONAL NITRIC OXIDE SYNTHASE**

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of Research Scientist Award MH-18501 awarded by the National Institutes of Health and USPHS DA0074.

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### **TECHNICAL AREA OF THE INVENTION**

The invention relates to the area of neurotransmitter regulation. More particularly, the invention relates to the regulation of neuronal nitric oxide synthase.

### **BACKGROUND OF THE INVENTION**

Studies of neuronally-derived nitric oxide (NO) have revealed many roles for this gaseous messenger molecule (Moncada, 1994; Yun et al., 1996). In the peripheral nervous system, NO mediates nonadrenergic, noncholinergic neurotransmission, serving as an effector of autonomic neurons on smooth muscle. NO has been implicated in several forms of neuronal plasticity, such as LTP (for a review see Huang, 1997). Studies in mice with a targeted genomic deletion of the NO biosynthetic enzyme, neuronal NO synthase (nNOS), have shown that NO mediates a substantial portion of the neurotoxicity associated with stroke (Huang et al., 1994). In the brain, NO and citrulline are produced from arginine predominantly by a neuronal isoform of NOS (nNOS) (Huang et al., 1993), although endothelial NOS (eNOS) may also occur in neurons (Dinerman et al., 1994; O'Dell et al., 1994). Most neurotransmitters are

5 stored in synaptic vesicles and neurotransmitter effects are elicited following the exocytosis of transmitter into the synaptic space. For an evanescent transmitter such as NO there is no storage pool and newly synthesized NO is used as it is made. NO synthesis is triggered by the influx of calcium, which, when complexed with calmodulin, activates the biosynthetic activity of NOS (Bredt and Snyder, 1990).

10 Because NO lacks vesicular storage and depends on new synthesis for its release, nNOS must be associated with the plasma membrane. Subcellular fractionation indicates that roughly half of brain nNOS is soluble and half particulate (Bredt, 1996; Hecker et al., 1994). Recently, Bredt and associates showed that nNOS is targeted to membranes by binding to syntrophin, PSD95/SAP90, or PSD93 (Brennan et al., 1996; Brennan et al., 1996). These proteins are enriched in synaptic densities and interact with nNOS through PDZ domains, consensus sequences of about 100 amino acids that are found in proteins which tend to be associated with cell-cell junctions (Ponting and Phillips, 1995). The nNOS/PSD95 interaction involves a portion of nNOS which includes its sole PDZ domain and the second PDZ domain of PSD95. PSD95 was first isolated from postsynaptic densities (Cho et al., 1992) but also occurs in presynaptic nerve terminals (Kistner et al., 1993) and clusters neurotransmitter receptors and ion channels at synaptic sites (Kornau et al., 1997). For instance, the NMDA receptor and several potassium channels are associated with PSD95 at synapses (Kornau et al., 1995). The linking of NMDA receptors to nNOS by PSD95 may explain why calcium influx following NMDA receptor activation leads to a tightly coupled nNOS activation (Brennan et al., 1996). Indeed, the effects of NO appear to be intimately tied to the NMDA receptor. For example, NMDA receptor-mediated neurotoxicity (Dawson and Dawson, 1996), neurotransmitter release (Schuman and Madison, 1994), and cGMP elevations (Bredt and Snyder, 1989; Garthwaite et al., 1989) each require nNOS and are blocked by nNOS-specific inhibitors. Moreover, NO can directly modulate NMDA receptors (Lipton and Stamler, 1994).

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30 There is a continuing need in the art of neurotransmitter regulation for methods of affecting the activity of neuronal NOS, so that one can manipulate NO levels when required for therapeutic effect in such disorders.

**SUMMARY OF THE INVENTION**

It is an object of the invention to provide an isolated mammalian Capon (Carboxy-terminal PDZ ligand of nNOS) protein.

5

It is another object of the invention to provide a fusion protein comprising at least eight contiguous amino acids selected from the Capon amino acid sequence shown in SEQ ID NO:2.

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It is yet another object of the invention to provide an isolated polypeptide consisting of at least eight contiguous amino acids of Capon as shown in SEQ ID NO:2 and capable of binding an nNOS PDZ domain.

15

It is still another object of the invention to provide a preparation of antibodies which specifically bind to a Capon protein as shown in SEQ ID NO:2 or 4.

It is even another object of the invention to provide a subgenomic polynucleotide which encodes a Capon protein as shown in SEQ ID NO:2 or 4.

It is yet another object of the invention to provide a recombinant DNA construct for expressing Capon antisense nucleic acids.

It is still another object of the invention to provide a method of inhibiting a mammalian neuronal nitric oxide synthase (nNOS).

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It is even another object of the invention to provide methods of screening test compounds for the ability to decrease or augment the activity of nNOS.

These and other objects of the invention are provided by one or more of the embodiments described below. One embodiment of the invention provides an isolated mammalian Capon protein which has the sequence shown in SEQ ID NO:2 or 4, and naturally occurring biologically active variants thereof.

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Another embodiment of the invention provides a mammalian Capon fusion protein which comprises two protein segments fused to each other by means of a peptide bond, wherein one of the protein segments consists of at least eight contiguous amino acids selected from the amino acid sequence shown in SEQ ID NO:2 or 4.

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Yet another embodiment of the invention provides an isolated polypeptide which consists of at least eight contiguous amino acids of Capon as shown in SEQ ID NO:2 or 4, wherein the polypeptide binds to an nNOS PDZ domain.

Still another embodiment of the invention provides a preparation of antibodies which specifically bind to a mammalian Capon protein as shown in SEQ ID NO:2 or 4.

Even another embodiment of the invention provides a subgenomic polynucleotide which encodes a Capon protein as shown in SEQ ID NO:2 or 4.

Yet another embodiment of the invention provides a recombinant DNA construct for expressing Capon antisense nucleic acids, comprising a promoter and a coding sequence for Capon consisting of at least 12 contiguous base pairs selected from SEQ ID NO:1 or 3, wherein the coding sequence is in an inverted orientation with respect to the promoter, such that upon transcription from the promoter an RNA is produced that is complementary to native mRNA encoding Capon.

Still another embodiment of the invention provides a method of decreasing a mammalian nNOS activity, comprising the step of contacting a nNOS with a Capon protein having an amino acid sequence as shown in SEQ ID NO:2 or 4.

Even another embodiment of the invention provides a method of screening test compounds for the ability to decrease or augment nNOS activity. The method comprises the steps of: (a) contacting a test compound with a mixture of a mammalian Capon protein and a polypeptide comprising an nNOS PDZ domain; and (b) measuring the amount of Capon or the polypeptide that is bound or unbound in the presence of the test compound, a test compound that decreases the amount of bound Capon or the polypeptide being a potential drug for increasing nNOS activity, and a test compound that increases the amount of the polypeptide or Capon that are bound being a potential drug for decreasing nNOS activity.

Yet another embodiment of the invention provides a method of screening test compounds for the ability to decrease or augment nNOS activity comprising the steps of: (a) contacting a cell with a test compound, wherein the cell comprises: i) a first fusion protein comprising (1) a DNA binding domain or a transcriptional activation domain, and (2) all or a portion of a mammalian Capon protein, wherein the portion consists of a contiguous sequence of amino acids selected from the amino acid sequence shown in SEQ ID NO:2 or 4, wherein the portion is capable of binding to nNOS; ii) a second fusion protein comprising (1) a transcriptional activation domain

or a DNA binding domain and (2) all or a portion of nNOS, wherein the portion comprises a PDZ domain, or a naturally occurring biologically active variant thereof, wherein the interaction of the portion of the Capon protein with the portion of nNOS reconstitutes a sequence-specific transcriptional activating factor, wherein when the 5 first fusion protein comprises a DNA binding domain the second fusion protein comprises a transcriptional activation domain and when the first fusion protein comprises a transcriptional activation domain the second fusion protein comprises a DNA binding domain; and iii) a reporter gene comprising a DNA sequence to which the DNA binding domain of the first fusion protein specifically binds; and (b) 10 measuring the expression of the reporter gene, a test compound that increases the expression of the reporter gene being a potential drug for decreasing nNOS activity, and a test compound that decreases the expression of the reporter gene being a potential drug for augmenting nNOS activity.

Even another embodiment of the invention provides a method of screening test 15 compounds for the ability to decrease or augment nNOS activity comprising the steps of: (a) contacting a cell with a test compound, wherein the cell comprises: (i) a first expression vector comprising a subgenomic polynucleotide encoding at least the PDZ domain of nNOS or a naturally occurring biologically active variant thereof; (ii) a second expression vector comprising a subgenomic polynucleotide encoding at least 20 the portion of Capon or a naturally occurring biologically active variant thereof, wherein the portion of Capon is capable of binding to nNOS; and (b) measuring the amount of cGMP, NO, or citrulline in the cell, a test compound that increases the amount of cGMP, NO, or citrulline being a potential drug for augmenting nNOS activity, and a test compound that decreases the amount of cGMP 25 being a potential drug for decreasing nNOS activity.

According to still another embodiment a method is provided for diagnosing a neurological disease or a propensity for a neurological disease, comprising: determining number of glutamine repeats present in a Capon protein of a patient wherein a number greater than 6 indicates a neurologic disease or a propensity therefor.

According to still another embodiment a method is provided for diagnosing a neurological disease or a propensity for a neurological disease, comprising: determining number of CAG repeats in a *Capon* gene of a patient, wherein a number greater than 6 indicates a neurologic disease or a propensity therefor.

5 Another aspect of the invention is a cell comprising one or more recombinant nucleic acid molecules. The cell comprises: (i) a first expression vector comprising a subgenomic polynucleotide encoding at least the PDZ domain of nNOS or a naturally occurring biologically active variant thereof, and (ii) a second expression vector comprising a subgenomic polynucleotide encoding at least a portion of *Capon* or a naturally occurring biologically active variant thereof, wherein the portion of *Capon* 10 is capable of binding to nNOS.

15 Another aspect of the invention is a cell comprising one or more recombinant nucleic acid molecules. The cell comprises: i) a nucleotide construct encoding a first fusion protein comprising (1) a DNA binding domain or a transcriptional activation domain, and (2) all or a portion of a mammalian *Capon* protein, wherein the portion consists of a contiguous sequence of amino acids selected from the amino acid sequence shown in SEQ ID NO:2 or 4, wherein the portion is capable of binding to nNOS; ii) a nucleotide construct encoding a second fusion protein comprising (1) a transcriptional activation domain or a DNA binding domain and (2) all or a portion of 20 nNOS, wherein the portion comprises a PDZ domain, or a naturally occurring biologically active variant thereof, wherein the interaction of the portion of the *Capon* protein with the portion of nNOS reconstitutes a sequence-specific transcriptional activating factor, wherein when the first fusion protein comprises a DNA binding domain the second fusion protein comprises a transcriptional activation domain, and when the first fusion protein comprises a transcriptional activation domain the second 25 fusion protein comprises a DNA binding domain; and iii) a reporter gene comprising a DNA sequence to which the DNA binding domain of the first fusion protein specifically binds, wherein upon reconstitution of the sequence specific transactivating factor, expression of the reporter gene is increased.

30 The present invention thus provides the art with the information that *Capon*, a heretofore unknown protein, regulates the activity of neuronal nitric oxide synthase.

Capon can be used, *inter alia*, in assays to screen for substances which have the ability to decrease or augment neuronal nitric oxide synthase activity.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

##### **Figure 1. Cloning of the CAPON cDNA and distribution of CAPON mRNA.**

5 (Figure 1 A) CAPON specifically interacts with nNOS in the yeast two-hybrid system. Yeast were transformed with the indicated Gal4 activation domain (AD) and Gal4 DNA-binding domain (BD) plasmids and grown on plates containing histidine. A typical filter lift is shown in which  $\beta$ -galactosidase activity was detected by the appearance of a blue precipitate. pAD-CAPON1, comprising the last 125 amino acids of CAPON, activates lacZ transcription in the presence of pBD-nNOS (2-377) but not with plasmids the first three PDZ domains of PSD95 (amino acids 20-364) or the second PDZ domain of PSD93 (amino acids 116-421).

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(Figure 1 B) Amino acid sequence of rat CAPON and alignment with a partial human sequence. The underlined sequence corresponds to the putative PTB domain. The bracketed sequence is encoded by a CAG repeat. A human expressed sequence tag (EST) (accession number R19867) obtained from a library derived from infant brain contains a 459 bp open reading frame with homology to the C-terminus of the CAPON cDNA ( $p = 5.1 \times 10^{-17}$ ). The conceptual translation of this clone reveals a protein with 92% amino acid identity with the rat protein.

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(Figure 1 C) CAPON is enriched in neuronal structures. Northern (RNA) blot analysis reveals that several CAPON transcripts are present, and these transcripts are enriched in neuronal tissues.

##### **Figure 2. Interaction of CAPON and nNOS.**

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(Figure 2 A) CAPON binds to nNOS, but not eNOS or iNOS. Bacterially expressed GST, GST-PIN, and GST-CAPON were bound to glutathione agarose and then incubated with lysates of HEK293 cells transfected with expression plasmids for the indicated isoforms of NOS. After extensive washing of the resins, bound NOS was detected with isoform specific antibodies. While nNOS binds to both GST-PIN and GST-CAPON, neither eNOS or iNOS bind to either protein. Input = 10% of starting material applied to each resin.

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**(Figure 2 B)** A GST-NOS fusion protein specifically binds to rat brain CAPON. A fusion protein consisting of GST and amino acids 1-100 of nNOS was bound to glutathione agarose and then incubated with cerebellar supernatants. After extensive washing, CAPON is detected on the NOS resin but not on the control GST resin. A second ~48 kD band is also detected with this antibody, but fails to interact with nNOS. This band may represent a cross-reactive protein, an alternatively spliced isoform of CAPON, or a degradation product.

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**(Figure 2 C)** CAPON interacts with nNOS directly. HEK293 lysates transfected with a expression plasmid containing the nNOS cDNA or empty vector were resolved by electrophoresis, transferred to nitrocellulose, and probed with radiolabeled CAPON (see Methods). Purified nNOS is recognized with this probe, along with a comigrating band detected in NOS-transfected but not mock-transfected cells.

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**(Figure 2 D)** CAPON and nNOS complexes are detectable in cerebellar lysates (I). Cerebellar supernatants were prepared from wild-type (+/+) and nNOS knockout (-/-) mice and incubated with the 2', 5', ADP-sepharose, an nNOS-affinity resin. Only CAPON derived from supernatants of wild-type and not knockout animals was capable of binding the resin indicating that the presence of nNOS is required for CAPON to bind to the resin. CAPON levels were decreased in knockout animals, presumably due to decreased stability in the absence of the nNOS binding partner. A lower molecular weight band (arrowhead), frequently detected with the anti-CAPON antibody, bound weakly to the resin in an nNOS-specific manner as well. Input = 20% of lysate used for binding.

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**(Figure 2 E)** CAPON and nNOS complexes are detectable in cerebellar lysates (II). An antibody to CAPON (5 mg) specifically coprecipitates nNOS, while comparable amounts of antibody to the G-protein subunit b1, cyclin-dependent kinase 2, and preimmune serum fail to coprecipitate nNOS. Enrichment of nNOS in CAPON immunoprecipitates was specific as a control protein, protein kinase C- b I/II did not display similar enrichment. Input = 10% of lysate used for immunoprecipitation.

**Figure 3.** Immunohistochemical localization of nNOS and CAPON.

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**(Figure 3 A)** Comparison of nNOS-IR (immunoreactivity), CAPON-IR, and CAPON in situ hybridization patterns in sagittal sections of adult rat. Islands of Calleja (solid arrowhead); supraoptic nucleus (open arrowhead); AOB, accessory olfactory bulb; C, colliculi; Cb, cerebellum; Cx, cerebral cortex; OB, olfactory bulb. Immunohistochemical nonspecific labelling ("Block") was determined using a CAPON antibody pre-absorbed with the antigenic peptide. Nonspecific hybridization ("Sense") was detected using a sense probe.

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**(Figure 3 B)** Comparison of cellular localization of nNOS-IR and CAPON-IR in adult rat brain. CAPON-IR (*a*) and nNOS-IR (*b*) hypothalamic neurons, solid arrowheads indicate IR dendritic processes. CAPON-IR (*c*) and nNOS-IR (*d*) of the supraoptic nucleus. CAPON-IR (*e*) and nNOS-IR (*f*) cell bodies of the nucleus of the trapezoid body separated by unreactive fascicles of nerve fibers. Adjacent is the pontine nucleus (Pn) which exhibits both CAPON and nNOS-IR (*e, f*). CAPON-IR (*g*) and nNOS-IR (*h*) in the cerebellum. Molecular cell layer (Mol); granular cell layer (Gr). Micrographs *e, f*, (100x); *c, d, g, h*, (200x); *a, b* (400x).

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**Figure 4.** The PDZ domain of nNOS interacts with the C-terminus of CAPON.

**(Figure 4 A)** The PDZ domain of nNOS (amino acids 20-100) is sufficient for binding to CAPON. Truncations of nNOS were subcloned into the Gal4 BD vector and nNOS/CAPON interactions were detected by  $\beta$ -galactosidase assays.

**(Figure 4 B)** The C-terminal 13 amino acids of CAPON are sufficient for binding to nNOS. Various GST-CAPON fusion proteins were incubated with HEK293 lysates containing nNOS. A CAPON fusion protein comprising the last 100 amino acids binds nNOS, as do fusion proteins comprising the last 13 or 20 amino acids of CAPON. A CAPON fusion protein with the last 20 amino acids deleted no longer binds nNOS.

Neither of the control proteins, GST or GST-14-3-3, are able to bind nNOS.

**(Figure 4 C)** Amino acid substitutions in the C-terminus of CAPON prevent it from interacting with nNOS. His<sub>6</sub>-fusion proteins of the last 100 amino acids of CAPON were generated and incubated with GST-NOS (1-100) immobilized on glutathione agarose. While the unmutagenized sequence binds (last four amino acids EIAV), mutation of the terminal valine (EIAA) or the penultimate alanine (EIDV) prevents

binding. Serine or alanine mutations are tolerated at the n-2 position (ESAV and EAAV), but truncation of the C-terminal 13 amino acids blocks binding altogether.

**Figure 5. CAPON and PSD95 compete for binding to nNOS.**

(Figure 5 A) His<sub>6</sub>-CAPON fusion proteins specifically block the nNOS/PSD95 interaction in vitro. The C-terminal 100 amino acids were fused to a His<sub>6</sub> tag and added to HEK293 lysates transfected with nNOS expression plasmids at the indicated fusion protein concentration. This mixture was added to GST-PSD95 (amino acids 20-364) or GST-PSD93 (116-421), the regions of these proteins previously shown to interact with nNOS (Brenman et al., 1996). The disruption of the nNOS/PSD95 interaction required the C-terminal 13 amino acids, as this fusion protein (DC20) fails to block the interaction even at 5 mM. Other control proteins such as His<sub>6</sub>-PIN or His<sub>6</sub>-FKBP do not disrupt the nNOS-PSD95 interaction.

(Figure 5 B) Quantification of CAPON inhibition of the nNOS/PSD95 interaction. HEK293 cells were transfected with nNOS expression plasmids and then metabolically labeled with [<sup>35</sup>S] methionine. Radiolabeled nNOS was purified and mixed with His<sub>6</sub>-CAPON as in (A), above. Bound nNOS was resolved by electrophoresis and counts were determined on a PhosphoImager.

**Figure 6. CAPON expression prevents the interaction of PSD95 and nNOS.**  
HEK293 cells were transfected with various combinations of expression plasmids for HA-tagged nNOS (HA-NOS), myc-tagged PSD95, or CAPON. Following transfection, the lysates were immunoprecipitated with an anti-HA antibody and bound proteins were detected with the appropriate antibodies. Following cotransfection of HA-NOS and myc-PSD95, myc-PSD95 is detected in anti-HA immunoprecipitates. Cotransfection of a full-length CAPON expression plasmid substantially reduces the amount of myc-PSD95 in anti-HA precipitates. In the absence of HA-nNOS transfection, neither myc-PSD95 or CAPON is immunoprecipitated by anti-HA antibodies.

**Figure 7. Model of PSD95/nNOS regulation by CAPON.**  
NMDA receptors are coupled to nNOS through a PSD95 multimer. These interactions are mediated by PDZ domains. In this complex, nNOS is situated close to NMDA receptor-modulated calcium influx (left). Binding of CAPON (right) results in a

reduction of NMDA receptor/PSD95/nNOS complexes, leading to decreased access to NMDA receptor-gated calcium influx and a catalytically inactive enzyme.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 We conducted a yeast two-hybrid screen in which we have identified a novel protein which we designate CAPON (Carboxyl-terminal PDZ ligand of nNOS). CAPON is a cytoplasmic protein whose carboxyl terminus binds to the PDZ domain of nNOS. CAPON competes with PSD95 and PSD93 for binding to nNOS and thus may participate in the translocation and impede the activation of this enzyme.

10 It is a discovery of the present invention that the mammalian protein Capon (Carboxy-terminal PDZ ligand of nNOS) physically interacts with and inhibits the activity of neuronal nitric oxide synthase (nNOS). Although it was known that nNOS regulates the release of its product, the messenger molecule nitric oxide, all of the proteins involved in its cellular localization were previously unknown.

15 Mammalian Capon protein has the sequence disclosed in SEQ ID NO:2 or 4, or other sequences which are at least about 80, 85, 87, 89, or 90% identical. Any biologically active variants of this sequence that may occur in mammalian tissues are within the scope of this invention. Biologically active variants bind to and inhibit nNOS binding to PSD95 and PSD93. Mammalian Capon proteins may comprise amino acids 1-503 as shown in SEQ ID NO:2 or 1-156 as shown in SEQ ID NO: 4. 20 Fragments of a mammalian Capon protein, comprising at least eight, nine, ten, twelve, thirteen, or sixteen consecutive amino acids selected from the sequence shown in SEQ ID NO:2 or 4, may also be used. Such fragments may be useful, for example, in various assays, as immunogens, or in therapeutic compositions. They may also be used as preparative reagents for purifying nNOS. A fusion protein may also be used for 25 many of these purposes, including as a reagent and as an immunogen.

30 A fusion protein consists of a full length mammalian Capon protein or a Capon protein fragment fused to a second protein or protein fragment by means of a peptide bond. The second protein or protein fragment may be, for example, a ligand for yet a third molecule. The second protein or protein fragment may be labeled with a detectable marker or may be an enzyme that will generate a detectable product. A

fusion protein may be useful, for example, to target full-length Capon protein or a Capon fragment comprising one or more specific domains, to a specific location in a cell or tissue.

Any of these Capon-related proteins may be produced by expressing Capon cDNA sequences in prokaryotic or eukaryotic host cells, using known expression vectors. Synthetic chemistry methods can also be used to synthesize Capon protein, fusion protein, or fragments. Alternatively, Capon protein can be extracted, using standard biochemical methods, from Capon-producing mammalian cells, such as brain cells. The source of the cells may be any mammalian tissue that produces Capon protein including human, rat, or mouse. Methods of protein purification, such as size exclusion chromatography, ammonium sulfate fractionation, ion exchange chromatography, affinity chromatography, or preparative gel electrophoresis, are well known in the art. Given the sequences disclosed in SEQ ID NO:2 and 4, an ordinary artisan can readily select appropriate methods to obtain a preparation of mammalian Capon protein that is substantially free from other mammalian proteins. An isolated Capon protein is purified from other compounds that may normally associate with Capon protein in a cell, such as certain proteins, carbohydrates, lipids, or subcellular organelles.

The present invention also provides a preparation of antibodies that specifically bind to mammalian Capon protein. The antibodies may be polyclonal or monoclonal and may be raised against biochemically isolated, chemically synthesized, or recombinantly produced full-length Capon protein, Capon protein fragments, or Capon fusion proteins. Techniques for raising antibodies directed against intracellular proteins such as mammalian Capon are well known in the art. The antibodies bind specifically to Capon epitopes, preferably epitopes not present on other mammalian proteins. Antibodies that bind specifically to Capon proteins include those that bind to full-length Capon protein, Capon fragments or degradation products, as well as to alternatively spliced forms of Capon proteins, or to Capon fusion proteins. In preferred embodiments of the invention the antibodies prevent Capon binding to nNOS, immunoprecipitate Capon protein from solution, and react with Capon protein on Western blots of polyacrylamide gels. Preferably the antibodies do not exhibit

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nonspecific cross-reactivity with other mammalian proteins on Western blots or in immunocytochemical assays. Techniques for purifying Capon antibodies are those which are available in the art. In a more preferred embodiment, antibodies are affinity purified by passing antiserum over a support column to which Capon protein is bound and then eluting the bound antibody, for example with high salt concentrations. Any such techniques may be chosen to achieve the preparation of the invention.

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The polynucleotides of the present invention encode Capon protein. These polynucleotides may be isolated and purified free from other nucleotide sequences by standard purification techniques, using restriction enzymes to isolate fragments comprising the Capon encoding sequences. The polynucleotide molecules are preferably intron-free and have the sequence shown in SEQ ID NO:1 or 3. Such Capon cDNA molecules can be made *inter alia* by using reverse transcriptase with Capon mRNA as a template. The polynucleotide molecules of the invention can also be made using the techniques of synthetic chemistry given the sequence disclosed herein. The degeneracy of the genetic code permits alternate nucleotide sequences to be synthesized that will encode the Capon amino acid sequence shown in SEQ ID NO:2 or 4. All such nucleotide sequences are within the scope of the present invention, as well as those which are at least 70, 75, 80, 85, or 90% identical. The Capon polynucleotide molecules can be propagated in vectors and cell lines as is known in the art. The constructs may be on linear or circular molecules. They may be on autonomously replicating molecules or on molecules without replication sequences. Recombinant host cells can be formed by introducing the genetic constructs of the present invention into cells. Any of those techniques which are available in the art can be used to introduce genetic constructs into the cells. These include, but are not limited to, transfection with naked or encapsulated nucleic acids, cellular fusion, protoplast fusion, viral infection, and electroporation. Introduction of genetic constructs may be carried out *in vitro* or *in vivo*.

The invention also provides a recombinant DNA construct for expressing Capon antisense nucleic acids. The construct contains a promoter and a coding sequence for Capon consisting of at least 12 and preferably at least 15 or 20 contiguous base pairs selected from SEQ ID NO:1 or 3. The Capon coding sequence

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is in an inverted orientation with respect to the promoter, so that when the sequence is transcribed from the promoter, an RNA complementary to native Capon-encoding mRNA is produced. The construct may also include a terminator at the 3' end of the inverted Capon coding sequence. The antisense molecules produced using the DNA construct of the invention may be used to decrease or prevent the transcription of Capon mRNA. The antisense molecules may be used *in vitro* or *in vivo*, as pharmacological agents for the purpose of influencing nNOS activity.

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According to the present invention, nNOS is inhibited by mammalian Capon protein, which competes with PSD95 and PSD93 for binding to nNOS, thereby inhibiting nNOS activity. Suitable inhibitory concentrations range from 1 nM to 1 mM. In a preferred embodiment the concentration of Capon protein is at least 250 nM. In a more preferred embodiment the concentration of Capon protein is at least 1  $\mu$ M. Greater concentrations of Capon protein may also be used. nNOS activity may be measured, for example, by assaying nitric oxide-dependent cGMP formation in HEK 293 cells cotransfected with DNA encoding Capon and nNOS. Other cell lines, such as mouse N1E-115 neuroblastoma cells, may be used as well. Formation of cGMP may be measured, for example, by radioimmunoassay or by spectrophotometry. nNOS activity may be measured in intact cells or in cell lysates. Other assays for measuring nNOS activity may also be used. NO or citrulline can also be measured.

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The present invention also provides methods of screening test compounds for the ability to decrease or augment nNOS activity. The test compounds may be pharmacologic agents already known in the art or may be compounds previously unknown to have any pharmacological activity. The compounds may be naturally occurring or designed in the laboratory. They may be isolated from microorganisms, animals, or plants, and may be produced recombinantly, or synthesized by chemical methods known in the art. A test compound can be contacted with a mixture of mammalian Capon protein (or the NOS-binding portion thereof) and a polypeptide containing an nNOS PDZ domain which is a contiguous sequence selected from the N-terminal about 100 amino acids of nNOS amino acid sequences as shown in SEQ ID NO:5 and 6. Analogous domains in other mammalian nNOS proteins can also be used. These are referred to as biologically active, naturally occurring variants of the rat

or human protein. These molecules may be produced recombinantly or may be synthesized using standard chemical methods. The nNOS or Capon binding partner may consist of less than the entire nNOS. The two binding partners may be prebound prior to the step of contacting with the test compound. Alternatively, the test compound may contact one of the binding partners before the second binding partner is added. The PDZ domain-containing molecule may be in solution or may be bound to a solid support. These molecules may be unlabeled or labeled, for example, with a radioactive, fluorescent, or other detectable marker. They may be fusion proteins comprising a nNOS PDZ domain and another protein with or without a detectable enzymatic activity. The amount of at least one of the two binding partners that is bound or unbound in the presence of the test compound is then measured. A number of methods may be used to measure the amount of bound molecules. For example, the relative concentration of bound to unbound may be detected by examining the apparent molecular masses of the molecules by size exclusion chromatography or by polyacrylamide gel electrophoresis under non-reducing conditions. Other methods of measuring binding or dissociation of the molecules will readily occur to those of ordinary skill in the art and can be used. A test compound that decreases the amount of the polypeptide and Capon that are bound is a potential drug for increasing nNOS activity. A test compound that increases the amount of the polypeptide and Capon that are bound is a potential drug for decreasing nNOS activity.

According to the present invention a method is also provided of using the yeast two-hybrid technique to screen for test compounds that decrease or augment nNOS activity. The yeast two-hybrid technique is generically taught in Fields, S. and Song, O., *Nature* 340, 245-46, 1989. In a preferred embodiment, a cell is contacted with a test compound. The cell comprises a first fusion protein comprising a DNA binding domain and all or a portion of a mammalian Capon protein consisting of a contiguous sequence of amino acids selected from the amino acid sequence shown in SEQ ID NO:2 and capable of binding to nNOS (this typically requires the 13 carboxy terminal amino acids). The cell also comprises a second fusion protein comprising a transcriptional activating domain and all or a portion of nNOS, wherein the portion comprises a contiguous sequence of amino acids selected from amino acids 14-89 as

shown in SEQ ID NO:5 or 6 or naturally occurring biologically active variants thereof. Alternatively, the DNA binding domain and the transcriptional activating domains can be paired with the opposite proteins. The interaction of the portion of the Capon protein with the portion of nNOS reconstitutes a sequence specific transcriptional activating factor. A reporter gene is also present in the cell. The reporter gene comprises a DNA sequence to which the DNA binding domain of the first fusion protein specifically binds. When the Capon and nNOS regions are bound together, the DNA binding domain and the transcriptional activating domain will be in close enough proximity to reconstitute a transcriptional activator capable of initiating transcription of a detectable reporter gene in the cell. The expression of the reporter gene in the presence of the test compound is then measured. A test compound that increases the expression of the reporter gene is a potential drug for decreasing nNOS activity. A test compound that decreases the expression of the reporter gene is a potential drug for augmenting nNOS activity. Test compounds which increase nNOS activity are potential drugs for modulating aggressive behaviour, particularly aggressive sexual behavior. Test compounds which decrease nNOS activity are potential drugs for treating stroke patients and other neuronal degeneration which is mediated by NO.

Many DNA binding domains and transcriptional activating domains can be used in this system, including the DNA binding domains of GAL4, LexA, and the human estrogen receptor paired with the acidic transcriptional activating domains of GAL4 or the herpes virus simplex protein VP16 (See, e.g., G.J. Hannon *et al.*, *Genes Dev.* 7, 2378, 1993; A.S. Zervos *et al.*, *Cell* 72, 223, 1993; A.B. Votjet *et al.*, *Cell* 74, 205, 1993; J.W. Harper *et al.*, *Cell* 75, 805, 1993; B. Le Douarin *et al.*, *Nucl. Acids Res.* 23, 876, 1995). A number of plasmids known in the art can be constructed to contain the coding sequences for the fusion proteins using standard laboratory techniques for manipulating DNA (see, e.g., Example 1, below). Suitable detectable reporter genes include the *E. coli lacZ* gene, whose expression may be measured colorimetrically (see, e.g., Fields and Song, *supra*), and yeast selectable genes such as *HIS3* (Harper *et al.*, *supra*; Votjet *et al.*, *supra*; Hannon *et al.*, *supra*) or *URA3* (Le Douarin *et al.*, *supra*). Methods for transforming cells are also well known in the art. See, e.g., A. Hinnen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 75, 1929-1933, 1978. The test compound may

comprise part of the cell culture medium or it may be added separately. The tester cell need not be a yeast cell, but may be a bacterial, other fungal, or mammalian cell.

In another embodiment, a cell is contacted with a test compound. In this embodiment, the cell comprises (i) a first expression vector comprising a subgenomic polynucleotide encoding nNOS or a naturally occurring biologically active variant thereof, and (ii) a second expression vector comprising a subgenomic polynucleotide encoding a portion of Capon or a naturally occurring biologically active variant thereof. The portion of Capon is capable of binding to nNOS. NO production by the cell is then measured, for example by radioimmunoassay or by spectrophotometry. A test compound that increases the amount of NO produced by the cell is a potential drug for augmenting nNOS activity. A test compound that decreases the amount of NO in the cell is a potential drug for decreasing nNOS activity. NO production may be determined by assaying for cGMP or citrulline as well. A test compound which binds to nNOS at the Capon binding site may either inhibit Capon binding, thus favoring the interaction of nNOS and PSD95 or mimic Capon thus inhibiting the interaction of nNOS and PSD95.

Because expansion of glutamine repeats has been shown to be associated with neurodegenerative and other neurological diseases, the glutamine repeat in Capon is believed to be relevant to pathogenesis. Thus either the Capon protein or the gene encoding it can be examined for the number of glutamine residues in the glutamine repeat region, or the number of glutamine codons (CAG) in the CAG repeat region of the gene. Expansion to a number greater than 6 indicates a propensity for or the presence of a neurological disease, likely a neurodegenerative disease. As in other examples where this mechanism has been demonstrated, the degree of expansion is associated with the severity of disease. It is likely that the glutamine repeat region interacts with a binding partner, and the avidity of the interaction is governed by the degree of expansion of the glutamine repeats. Any means for determining the sequence of a gene or protein may be used, including but not limited to direct sequencing, hybridization with allele-specific probes, binding to antibodies, size determination on gel electrophoresis.

The main finding of this study is the identification of a novel protein, CAPON, which interacts selectively with nNOS. The interaction of CAPON with nNOS is highly specific and has been verified by several methods of monitoring protein-protein interactions. The similarities in neuronal localizations of CAPON and nNOS imply that these proteins interact physiologically and that the principal biological function of CAPON may be to interact with nNOS. The apparent selectivity of this interaction contrasts with other nNOS binding proteins such as PSD95, PIN and calmodulin, each of which bind to multiple other proteins.

The competitive binding for nNOS by CAPON and PSD95 suggests a model for regulating the translocation of nNOS between cytoplasm and synaptic structures (Figure 7). Presumably, NO release into the synaptic space must be preceded by its translocation to synaptic structures by binding to PSD95. We propose that this process can be blocked by CAPON's removal of nNOS from PSD95, and translocating nNOS into the cytoplasm, or some other cellular compartment. In this manner CAPON could lead to effective nNOS inhibition. Although CAPON does not inhibit nNOS catalytic activity directly (data not shown), CAPON would reduce the accessibility of nNOS to NMDA receptor-mediated calcium influx, thus diminishing the capacity of nNOS to exert its physiologic or pathologic effects. Small molecules which specifically bind to nNOS in a manner similar to that of CAPON are useful for blocking NO-mediated neuronal degeneration.

We have explored potential mechanisms that might regulate the nNOS/CAPON interaction. For instance, we phosphorylated nNOS in transfected HEK 293 cells by treatment with forskolin, phorbol esters, dibutyryl cyclic AMP and 8-bromo-cyclic GMP and in vitro or with purified nNOS protein utilizing protein kinase C, protein kinase A and calcium calmodulin dependent protein kinase using methods described previously (Bredt et al., 1992). We have been unable to alter CAPON-nNOS interactions by any of these treatments.

Conceivably, phosphorylation of CAPON regulates its interactions with nNOS. Recently some of us showed that phosphorylation of the n-2 serine in the potassium channel BIRK-2 regulates its binding to a PDZ domain in PSD95 (Cohen et al., 1996). CAPON, and several other PDZ-domain ligands (Songyang et al., 1997), lack a serine

in this position and so must be regulated in some other manner. One possible mechanism may be a regulation of the ligand's C-terminal secondary structure. A recent crystallographic study of a PDZ domain complexed with a short cognate peptide shows that the peptide binds in an antiparallel beta-sheet conformation, with characteristic beta-sheet contacts between the peptide and a strand of a beta-sheet within the PDZ domain (see Doyle et al., 1996). In a physiologic setting, the unbound cognate sequence may constitutively adopt a beta-sheet conformation, with the other beta-strands coming from other, possibly distant, residues within the ligand protein's sequence. This beta-sheet might constitute an endogenous high-affinity ligand. This notion is supported by our observation that short, presumably unfolded, peptides comprising the C-terminal nine residues of CAPON bind nNOS weakly, while 16-residue peptides are more potent competitors ( $IC_{50}=10$  mM), although both are much less effective than 100 amino acid fusion proteins which are active in the nanomolar range (see Figure 5). Peptide competitors that interact with other PDZ domains have also been utilized at 10 and 500 mM concentrations (see Brenman et al., 1996; Kornau et al., 1995). Conceivably, the nNOS-CAPON interaction would be disrupted simply by disrupting the beta-sheet conformation of the C-terminus, which might be achieved by phosphorylation at a distance.

The nNOS PDZ domain is the first example of a PDZ domain which binds to other PDZ domains. The region of nNOS which possesses this property is the PDZ domain plus the adjacent ~50 amino acids on the carboxyl-side of the PDZ domain (residues 1-150) (Brenman et al., 1996). The additional amino acids in this super-sized PDZ domain may be required to accommodate larger ligands such as other PDZ domains. The finding in this report of another physiologic ligand for the PDZ domain, namely, the C-terminal region of CAPON, raises the question of whether the same or different portions of the nNOS PDZ domain account for the binding to two seemingly different ligands. Because these interactions are mutually exclusive, it is likely that the ligand-binding cleft in the PDZ domain mediates both interactions. Previously identified proteins which contain C-terminal PDZ-binding sequences have been membrane associated. By contrast, CAPON is soluble. This demonstrates that PDZ domains may mediate purely cytosolic protein-protein interactions.

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Stricker et al. (1997) recently characterized the specificity of the nNOS PDZ-binding domain. These researchers used a phage display method to identify NOS-binding peptides. Peptides ending in the sequence aspartate-X-valine were found to be high affinity ligands. Interestingly, unlike CAPON, these peptides did not bind the canonical nNOS PDZ domain (amino acids 13-89) but bound the extended PDZ domain only (amino acids 1-150). This extended domain is the minimal sequence which mediates PDZ-PDZ interactions. Presumably the differences in the binding sites in nNOS for the phage display peptide and CAPON account for the differences between the sequence specificity requirements for PDZ-PDZ interactions and PDZ-CAPON interactions.

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The following are provided for exemplification purposes only and are not intended to limit the scope of the invention which has been described in broad terms above.

### **EXAMPLE 2.**

#### **Identification and Cloning of CAPON**

We conducted a yeast two-hybrid screen employing the first 377 amino acids of nNOS, a region which includes the PDZ domain that comprises the first 100 amino acids of nNOS. Screening of six million clones resulted in the identification of three distinct cDNA inserts, one of which, PIN, has been previously reported (Jaffrey and Snyder, 1996) while the other two are overlapping cDNAs derived from a gene which is designated CAPON. The CAPON two-hybrid clones share a common carboxyl terminus and are predicted to translate into 125 and 327 amino acid peptides followed by a stop codon. The 125 amino acid C-terminal fragment of CAPON specifically interacts with nNOS in the two-hybrid system as is evident from the failure of CAPON to interact with fragments of PSD93 and PSD95 containing PDZ domains (Figure 1A). Moreover, nNOS fails to interact with another control protein, c-fos. To obtain a full-length CAPON cDNA, we screened a rat brain cDNA library with the larger two-hybrid clone and isolated a 2100 bp cDNA which overlapped with the two-hybrid clone and was used to assemble a final 2820 bp cDNA (see Methods). The conceptual translation of this cDNA produces a 503 amino acid protein (Figure 1B). The first ATG in the cDNA was 393 bp from the 5' end of the cDNA and was situated in a

context that conformed to the Kozak consensus sequence for an initiator methionine (Kozak, 1991).

CAPON displays no significant homology to any other known class of protein except for an N-terminal 145 amino acid stretch of amino acids which has residues suggestive of a phosphotyrosine-binding (PTB) domain (Zhou et al., 1995). CAPON's PTB domain most closely resembles the mouse numb protein's PTB domain (Zhong et al., 1996) with nearly 28% sequence identity on this region. The similarity between CAPON and numb are limited to this domain. PTB domains are targeted to phosphotyrosine containing proteins such as growth factor receptors (reviewed in van 5  
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der Geer and Pawson, 1995).

Outside of the PTB domain, CAPON lacks any well known consensus sequences except for an 18 nucleotide stretch of CAG repeats that corresponds to six glutamines. Glutamine repeats occur in proteins whose expansion results in neurodegenerative diseases as exemplified by huntingtin, the protein which is altered in Huntington's disease (Ross, 1995). A BLAST search (Altschul et al., 1990) of an expressed sequence-tag database, dBEST, reveals a human brain-derived EST with ~75% nucleotide identity to CAPON. The cDNA insert was 1.4 kb and corresponds to the C-terminal 156 amino acids of CAPON plus one kb of 3' UTR. The conceptual translation of this portion of human CAPON has 92% amino acid identity with the rat protein (Figure 1B).

Northern (RNA) blotting reveals a predominant 7.5 kb transcript which is detected only in brain regions with no expression evident in adrenal, bladder, heart, kidney, lung and skeletal muscle (Figure 1C). Marked regional variations occur in the brain with highest densities in the cerebral cortex and medulla-oblongata and lowest levels in the hippocampus.

To assess the specificity of interactions between CAPON and nNOS, we evaluated the binding of a GST-CAPON fusion protein, consisting of C-terminal 125 amino acids of CAPON, with nNOS, eNOS and inducible NOS (iNOS) (Figure 2A). Lysates of HEK-293 cells transfected with expression plasmids for each of the three forms of NOS were incubated with GST-CAPON. After extensive washing of the resin, bound nNOS was detected by Western blotting with the appropriate isoform-specific

antibody. nNOS interacts strongly with GST-CAPON, while eNOS and iNOS do not interact. No interactions are evident with the GST control.

We also examined interactions of CAPON and nNOS by utilizing a GST-fusion protein of the NH<sub>2</sub>-terminal 100 amino acids of nNOS, the PDZ domain. Following incubation with cerebellar lysates the washed GST-nNOS resin was subjected to SDS-PAGE and Western blotted with a purified antibody directed against the C-terminal 125 amino acids of CAPON. A single band corresponding to CAPON is detected, while no CAPON is bound to the GST control. A lower molecular weight band in homogenates is occasionally detected using our anti-CAPON antibodies. This protein fails to interact with GST-nNOS fusion proteins (Figure 2B) and may represent an unrelated cross-reactive protein, or the product of an alternatively spliced CAPON mRNA which fails to bind nNOS.

The protein-protein interactions we detected with cell lysates and GST-fusion proteins might have reflected a tertiary interaction between nNOS, an unidentified protein and CAPON. To determine if CAPON directly binds to nNOS, we conducted blot overlay experiments (Figure 2C). Lysates of HEK 293 cells transfected with nNOS were resolved on a polyacrylamide gel, transferred to nitrocellulose and then probed with [<sup>32</sup>P]GST-CAPON. The radiolabeled CAPON probe binds to a single 160 kD band comigrating with a nNOS standard, while no binding is evident in mock transfected cells, demonstrating that CAPON physically interacts in a direct manner with nNOS.

To ascertain if complexes of CAPON and nNOS exist physiologically, we used two approaches. Our first approach took advantage of a NOS-affinity resin consisting of 2', 5'-ADP ribose crosslinked to an agarose matrix. This resin has been used previously to purify nNOS from cerebellar supernatants (Bredt and Snyder, 1990). Following incubation with cerebellar supernatants, the resin and washed extensively. As expected, a significant portion of the nNOS found in the starting material was bound to the resin (Figure 2D). To determine if nNOS and CAPON were physiologically associated, we next assayed for CAPON bound to the resin. Like nNOS, CAPON was substantially enriched in the bound fraction. As a control we assayed for the resin-binding ability of CAPON from supernatants derived from mice

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with a genomic deletion of nNOS. These mice express a truncated version of nNOS which lacks the PDZ domain and is unable to bind CAPON. Substantially less CAPON in these supernatants bound to the 2', 5'-ADP ribose resin indicating that CAPON has negligible intrinsic affinity for the resin and that CAPON-binding in wild-type supernatants was due to nNOS. The smaller cross-reactive band was also enriched on this resin, supporting the notion that it is in some manner related to CAPON by alternative splicing or proteolytic degradation. Interestingly, the total level of CAPON in knockout supernatants was approximately one half that in wild-type mice, possibly due to the absence of a stabilizing effect of nNOS.

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As a second approach, we immunoprecipitated CAPON from cerebellar supernatants and assayed for nNOS by Western blot (Figure 2E). nNOS coprecipitates with anti-CAPON antibodies but is not detected in immunoprecipitates generated using preimmune serum, an antibody to the G-protein subunit  $\beta 1$ , or with an antibody to cyclin-dependent kinase 2. To determine if the enrichment observed in anti-CAPON immunoprecipitates was specific, we asked if a control protein, protein kinase C- $\beta$  I/II, was similarly enriched in these fractions. We found that this protein was absent in all of the precipitates (Figure 2E) indicating that the coprecipitation of nNOS with CAPON was specific. These two approaches support the notion that CAPON and NOS exist as a complex in the rat cerebellum.

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In other experiments we metabolically labeled N1E-115 mouse neuroblastoma cells with [ $^{35}$ S] methionine and examined for the presence of proteins that would bind to GST-CAPON. The only protein that specifically interacted with CAPON is a 160 kD protein that comigrates with an nNOS standard (unpublished observations), suggesting that nNOS is the most abundant CAPON-binding protein in these cells.

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### General Methods and Materials

Molecular biology reagents were from New England Biolabs (Beverly, MA) and all other reagents were from Sigma (St. Louis, MO) except as indicated. Protein concentrations were determined by Bradford assay.

### Yeast two-hybrid methods

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Two-hybrid screens and the construction of the parent vectors pPC86, containing the GAL4-activation domain, and pPC97, containing the GAL4-DNA binding domain,

have been described (Jaffrey and Snyder, 1996). Plasmid pBD-NOS(2-377) was prepared by the insertion of an nNOS PCR product corresponding to amino acids 2-377 of rat nNOS into the Sal I and Bgl II sites of pPC97, resulting in an open reading frame encoding a GAL4 BD-NOS fusion protein (Jaffrey and Snyder, 1996). The nNOS fragment was constructed by PCR using the following primers: 5'-GACTAGTCGACTGAAGAGAACACGTTGGG-3' (coding strand, SEQ ID NO: 7) and 5'-TCTGCAGATCTCAGTGGGCCTTGGAGCCAAA-3' (noncoding strand, SEQ ID NO: 8).

A rat hippocampal cDNA library in pPC86 (Li et al., 1995) was amplified once in DH10B (Gibco BRL) as described (Jaffrey and Snyder, 1996) and transformed into yeast containing the pBD-NOS(2-377) plasmid. pAD-CAPON1 and pAD-CAPON2 were identified as 0.8 kb and 1.9 kB clones, respectively, that activated lacZ transcription and conferred histidine prototrophy in the presence of pBD-NOS(2-377). Plasmids were sequenced by automated fluorescent sequencing of both strands. Yeast two-hybrid vectors containing the second PDZ domain of PSD93 (amino acids 116-421) and the three PDZ domains of PSD95 (amino acids 20-364) have been described previously (Brenman et al., 1996).

Truncated NOS fragments comprising amino acids 2-165 and 2-284 were generated by restriction of the initial NOS (2-377) PCR fragment with Nco I and Ava I, respectively followed by Klenow-filling in of that end and ligation into pPC97. Other truncated NOS fragments were prepared by PCR and have been described (Jaffrey and Snyder, 1996).

#### cDNA cloning of CAPON

A CAPON DNA probe was generated by the random hexamer method using the pAD-CAPON2 cDNA as a template. This probe was used to screen a rat brain lambdaZAP II cDNA library (Stratagene) using methods described by the manufacturer. A 2.1 kb cDNA was isolated which overlapped with the pAD-CAPON2 clone. The cDNAs were ligated at an overlapping Xba I site to produce the full-length 2,812 bp cDNA and subcloned into pCMV for eukaryotic expression. The human CAPON EST was the sole CAPON homolog identified in a BLAST seacrh (Altschul et al., 1990).

I.M.A.G.E. consortium (<http://www-bio.llnl.gov/bbrp/image/image.html>) clone 34183 (Lennon et al., 1996) was purchased from Research Genetics (Huntsville, AL).

#### RNA (Northern) blotting

5 Thirty micrograms of whole RNA were isolated from rat tissues using the Triazol reagent (Gibco BRL) and separated on agarose-formaldehyde gels. RNA was transferred to Hybond N+ membranes (Amersham) and a DNA probe, generated using the random hexamer method with the two-hybrid CAPON cDNA as a template, was hybridized in Rapid-hyb buffer (Amersham) overnight at 65°C. The blots were subsequently washed sequentially in 2X SSC with 0.1% SDS, once at room 10 temperature for 15 min, 1X SSC with 1% SDS twice at 65°C, 0.1XSSC with 1% SDS twice at 65°C, and then with 0.1X SSC with 5% SDS twice at 65°C. The blot was apposed to film for 4 days at -80°C to visualize the bands.

#### GST-fusion protein binding assays

15 GST-fusion proteins were prepared in BL21(DE3) Escherichia coli (Novagen) with glutathione agarose as an affinity resin for purification (Smith and Johnson, 1988), except that bacterial pellets were sonicated in lysis buffer (50 mM Tris-HCl (pH 7.7), 100 mM NaCl, and 2 mM EDTA), supernatants were adjusted to 1% Triton X-100, washes were done in 50 mM Tris-HCl (pH 7.7), 500 mM NaCl, 2 mM EDTA, and 1% Triton X-100, and protein was purified with elution buffer (50 mM tris-HCl (pH 7.7), 20 100 mM NaCl, 10 mM reduced glutathione, and 2 mM EDTA).

25 Transfections were performed with 10 µg of each plasmid using the calcium phosphate method. Following transfection, cells were sonicated in buffer A [50 mM Tris-HCl (pH 7.7), 100 mM NaCl, 2 mM EDTA, and 1% Triton X-100] and cleared by centrifugation. This cellular lysate was incubated with GST-fusion protein immobilized on glutathione-agarose for one hour at 4°C and washed extensively in HNTG buffer [20 mM Hepes (pH 7.4), 500 mM NaCl, 10% glycerol, and 0.1% Triton X-100] five times, for ten minutes per wash at room temperature. A GST-CAPON fusion protein consisting of amino acids 379-503 was used for binding assays because it was more soluble when expressed in bacteria than larger CAPON fusion proteins.

For quantitative binding experiments, transfected cells were metabolically labeled overnight with 200 mci [<sup>35</sup>S] methionine and nNOS was purified by NADPH elution of 2',5' ADP ribose as described previously (Bredt and Snyder, 1990).

5 The material remaining on the resin was eluted with SDS-PAGE sample buffer and nNOS was detected by immunoblot using antibodies specific to each NOS isoform (Transduction Labs). A polyclonal antiserum to CAPON was generated in rabbits by using a His<sub>6</sub>-tagged CAPON fusion protein. GST-CAPON was crosslinked to glutathione agarose with dimethylpimelimidate and this resin was used to purify CAPON antibody. To confirm the specificity of the antibody, immune serum was 10 incubated with His<sub>6</sub>-CAPON which results in the abolishment of the signal. Incubation with His<sub>6</sub>-FKBP has no effect on the signal (data not shown).

15 For blot-overlay analysis, CAPON was inserted into pGEX-4T2, a derivative of PGEX4T2 in which two cyclic AMP-dependent protein kinase (PKA) sites were inserted between the GST moiety and the multiple cloning site (Jaffrey and Snyder, 1996). Kinase reactions and blot overlays were performed as described (Kavanaugh and Williams, 1994).

#### EXAMPLE 2.

#### **The C-terminus of CAPON Binds to the PDZ Domain of nNOS**

20 To examine the region of nNOS that binds to CAPON, we conducted yeast two-hybrid experiments with various truncations of nNOS (Figure 4A). As little as the first 100 amino acids of nNOS binds to the C-terminal 125 amino acids of CAPON. This portion of nNOS contains the full PDZ domain as defined by MacKinnon and associates (Doyle et al., 1996) who identified the PDZ consensus domain in nNOS as amino acids 14-89. Deletion of the first 20 amino acids of nNOS, which includes the 25 first seven amino acids of the PDZ domain, does not abolish binding, but larger NH<sub>3</sub>-terminal deletions abolish binding, presumably because they result in a loss of important structural components of the PDZ domain. The nNOS construct comprising amino acids 163-245, which represents the PIN-binding domain of nNOS (Jaffrey and Snyder, 1996), shows no interaction with CAPON.

30 PDZ domains typically interact with a characteristic C-terminal motif in other proteins (Songyang et al., 1997). By contrast, the nNOS PDZ domain binds directly

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to other PDZ domains, such as those in PSD95, but has not previously been reported to interact with any known physiological C-terminal peptide motifs. Since we could not detect any PDZ domain motifs in the CAPON sequence we sought to determine the region in CAPON which accounted for nNOS binding. We investigated the domain of CAPON that interacts with nNOS using GST-CAPON fusion proteins containing various deletions at the C-terminus (Figure 4B). Immobilized fusion proteins were incubated with HEK 293 lysates containing nNOS. Bound nNOS was detected by Western blot. Robust interactions with nNOS are evident with constructs containing as little as the C-terminal thirteen amino acids of CAPON. Deleting the C-terminal 20 amino acids of CAPON abolishes its interactions with nNOS. These data show that the C-terminal portion of CAPON is necessary and sufficient for nNOS binding.

Recently Cantley and associates (Songyang et al., 1997) identified consensus sequences for binding to several PDZ domains. Binding of PDZ ligands involves the C-terminus of proteins, with determinants of specificity lying within the eight or fewer C-terminal amino acids. A consistent requirement among all the PDZ domain ligands is a hydrophobic residue, such as valine or leucine, as the final amino acid. To determine if the binding of CAPON to nNOS exhibits similar sequence-specificity, we examined the binding of mutagenized His<sub>6</sub>-CAPON fusion proteins to immobilized GST-nNOS PDZ domain fusion proteins (Figure 4C). The C-terminal residue of CAPON is a valine, and conversion of this residue to alanine abolishes binding. Binding is also greatly reduced by changing the penultimate amino acid from alanine to aspartate. However, changing the n-2 amino acid from isoleucine to serine or alanine does not alter binding. These experiments indicate that the nNOS-CAPON interaction resembles those of other PDZ-C-terminal peptide ligand interactions. Specifically, C-terminal residues of CAPON are important for the specificity in CAPON binding to nNOS.

#### EXAMPLE 2.

#### **CAPON and PSD95 Compete for Binding to nNOS**

Since the nNOS PDZ domain is capable of both PDZ-PDZ interactions and PDZ-C-terminal peptide interactions we wondered whether CAPON and PSD95 can

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bind simultaneously to nNOS or whether their interactions with nNOS are mutually exclusive. To answer this question we incubated lysates of HEK-293 cells containing nNOS with various concentrations of a His<sub>6</sub>-CAPON fusion protein, comprising the last 125 amino acids of CAPON, and then added these lysates to GST-PSD95 immobilized on glutathione agarose resin (Figure 5A). After extensive washing of the resin, we assayed for nNOS bound to PSD95 by Western blot. As little as 5 nM His<sub>6</sub>-CAPON causes a substantial reduction of nNOS binding to GST-PSD95. Half maximal reduction of binding is evident between 5 and 50 nM His<sub>6</sub>-CAPON (Figure 5B). Deletion of the C-terminal 20 amino acids of CAPON abolishes its ability to serve as a competitor for nNOS binding to GST-PSD95. As controls, we examined the effects of 5  $\mu$ M His<sub>6</sub>-PIN or 5 mM His<sub>6</sub>-FK506 binding protein-12 kD (FKBP). Neither of these proteins compete for binding to nNOS. nNOS also binds to the second PDZ domain of PSD93, a protein which is highly homologous to PSD95 (Brenman et al., 1996). His<sub>6</sub>-CAPON is an effective competitor for nNOS binding to immobilized GST-PSD93 as well (Figure 5A, B). These effects of CAPON likely reflect its binding to nNOS rather than to the PDZ domains of either PSD95 or PSD93 because CAPON fails to interact with either PSD93 or PSD95 using (i) a two-hybrid assay (see Figure 1A) and (ii) in vitro experiments utilizing immobilized GST-PSD95 and purified recombinant CAPON (data not shown).

We wanted to determine if CAPON and PSD95 compete for binding to nNOS in intact cells. Accordingly, we transfected HEK-293 cells with various mixtures of expression plasmids containing cDNAs of hemagglutinin antigen (HA)-tagged nNOS, myc-tagged PSD95 and/or full-length CAPON. Following immunoprecipitation with antibodies to HA, we examined which proteins coprecipitated. In cells expressing HA-nNOS and myc-PSD95, antibodies to HA coprecipitate myc-PSD95 (Figure 6). When various amounts of CAPON cDNA containing expression plasmids are also transfected, HA-immunoprecipitates contain CAPON but substantially less PSD95.

### Immunoprecipitations

Immunoprecipitations were performed by homogenizing one rat cerebellum in 3 ml lysis buffer followed by centrifugation at 100,000  $\times$  g for 30 min at 4°C. Two hundred

microliters of the supernatant was incubated with 40 ml of protein A-agarose (Oncogene Sciences, Cambridge, MA) and 5 mg of the indicated antibody for 60 min at 4°C. The resins were then washed with IP wash buffer (50 mM Tris-HCl (pH 7.7), 400 mM NaCl, and 2 mM EDTA) six times and eluted in 60 ul of 1X SDS-PAGE sample buffer by boiling. Western blots were performed using an nNOS-specific monoclonal antibody (Transduction Labs) or a PKC- b I/II monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The immunoprecipitating antibodies used as controls were from Santa Cruz Biotechnology, Inc.

For experiments utilizing 2', 5', ADP sepharose (Pharmacia), tissues were prepared identically as for immunoprecipitations except mouse cerebella were used and the homogenization volume was 400 ml per cerebellum. Supernatants were incubated with 100 ul of affinity resin. Incubations and washes were performed identically as for immunoprecipitations. The generation of mice with a targetted deletion of nNOS has been described previously (Huang et al., 1993).

## 15 Immunohistochemistry

Adult Sprague-Dawley rats (200-250 gm) were obtained from Charles River and housed at the Johns Hopkins Animal Care Facility. A polyclonal antiserum to the C-terminal region of human nNOS (residues 1419-1433) was kindly provided by J. Spangenberg (IncStar, Stillwater, MN). The peroxidase Elite staining kit was from 20 Vector Laboratories.

Anesthetized rats were perfused through the left ventricle with 50 ml of 0.9% NaCl followed by 500 ml 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed, cut into sagittal blocks, and postfixed in 4% paraformaldehyde in 0.1 M PB for 4 h at room temperature. Blocks were 25 cryoprotected for 2 days at 4°C in 50 mM sodium phosphate, pH 7.4/0.1 M NaCl/20% (vol./vol.) glycerol. Brain sections, 40 mm thick, were cut on a sliding microtome. Free-floating sections were incubated in PBS (10 mM, pH 7.4/0.19 M NaCl), containing 4% normal goat serum (Jackson Labs), and 0.2% Triton X-100 for 45 min, and then incubated overnight at 4°C with the primary antiserum diluted 1:500 30 (CAPON) or 1:15,000 (nNOS) in phosphate buffered saline (PBS) containing 2% goat

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serum and 0.1% Triton X-100. Immunoreactivity was visualized with the Vectastain ABC Elite kit following the nickel-enhanced diaminobenzidine procedure. To test immunohistochemical specificity of the CAPON antiserum, the antiserum was incubated overnight with 13.5 mg/ml of the antigenic fusion protein before incubation with brain sections.

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### In situ hybridization

In situ hybridization used DNA oligonucleotide probes corresponding to amino acids 478-503. Probes were end-labelled with [ $\alpha$ -<sup>32</sup>P] dATP and terminal deoxynucleotidyl transferase to a specific activity of 800 mCi/mg and in situ hybridization was performed as described previously (Jaffrey et al., 1994). Non-specific hybridization was determined using the corresponding sense probe.

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**SEQUENCE LISTING SUMMARY**

10 SEQ ID NO: 2. Rat capon cDNA  
SEQ ID NO: 2. Rat capon amino acids  
SEQ ID NO: 2. Human capon cDNA  
SEQ ID NO: 2. Human capon amino acids  
SEQ ID NO: 2. Rat nNOS amino acids  
SEQ ID NO: 2. Human nNOS amino acids  
15 SEQ ID NO: 2. nNOS probe  
SEQ ID NO: 2. nNOS probe

**CLAIMS**

1. An isolated mammalian Capon protein which binds to the PDZ domain of a mammalian nitric oxide synthase (nNOS).
2. The Capon protein of claim 1 which has the amino acid sequence shown in SEQ ID NO:2 or 4, and naturally occurring biologically active variants thereof.
- 5 3. The Capon protein of claim 1 which is made by isolating the protein from human cells.
4. The Capon protein of claim 1 which is made by isolating the protein from rat cells.
- 10 5. The Capon protein of claim 1 which is produced recombinantly.
6. The Capon protein of claim 1 which is produced by synthetic chemical methods.
- 15 7. A fusion protein which comprises a first protein segment and a second protein segment fused to each other by means of a peptide bond, wherein the first protein segment consists of at least eight contiguous amino acids selected from the amino acid sequence shown in SEQ ID NO:2 or 4.
8. The fusion protein of claim 7 wherein the second protein segment consists of glutathione-S-transferase.
- 20 9. An isolated polypeptide which consists of at least eight contiguous amino acids of Capon as shown in SEQ ID NO:2 or 4, wherein the polypeptide binds to a PDZ domain of a mammalian neuronal nitric oxide synthase (nNOS).
10. A preparation of antibodies which specifically bind to a Capon protein as shown in SEQ ID NO:2 or 4.
- 25 11. The preparation of antibodies of claim 10 wherein the antibodies are monoclonal.
12. The preparation of antibodies of claim 10 wherein the antibodies are purified from an animal antiserum.
13. The preparation of antibodies of claim 10 wherein the antibodies are affinity purified.

14. A subgenomic polynucleotide which encodes a Capon protein as shown in SEQ ID NO:2 or 4.
15. The subgenomic polynucleotide of claim 14 which is intron-free.
16. The subgenomic polynucleotide of claim 15 which comprises the sequence shown in SEQ ID NO:1 or 3.
17. A vector comprising the polynucleotide of claim 14.
18. A vector comprising the polynucleotide of claim 15.
19. A vector comprising the polynucleotide of claim 16.
20. A recombinant host cell which comprises the vector of claim 17.
- 10 21. A recombinant host cell which comprises the vector of claim 18.
22. A recombinant host cell which comprises the vector of claim 19.
23. A recombinant DNA construct for expressing Capon antisense nucleic acids, comprising:
  - a promoter; and
  - 15 a coding sequence for Capon consisting of at least 12 contiguous base pairs selected from SEQ ID NO:1 or 3, wherein the coding sequence is in an inverted orientation with respect to the promoter, such that upon transcription from said promoter an RNA is produced which is complementary to native mRNA encoding Capon.
- 20 24. The construct of claim 23 further comprising a transcription terminator, wherein the coding sequence is between the promoter and the terminator.
- 25 25. A method of inhibiting the activity of a mammalian neuronal nitric oxide synthase (nNOS), comprising the step of:
  - contacting a nNOS with a Capon protein having an amino acid sequence as shown in SEQ ID NO:2 or 4 or a naturally occurring biologically active variant thereof, wherein the Capon protein is present in an inhibitory-effective amount.
26. The method of claim 25 wherein said Capon protein is present at a concentration of at least 250 nM.
- 30 27. The method of claim 25 wherein the Capon protein is present at a concentration of at least 1  $\mu$ M.

28. A method of screening test compounds for the ability to decrease or augment nNOS activity, comprising the steps of:

5 (a) contacting a test compound with all or a portion of a mammalian Capon protein and a polypeptide comprising an nNOS PDZ domain, wherein the Capon protein is capable of binding to nNOS; and

10 (b) measuring the amount of Capon or the polypeptide that is bound or unbound in the presence of the test compound, a test compound that decreases the amount of bound Capon or the polypeptide being a potential drug for increasing nNOS activity, and a test compound that increases the amount of the polypeptide or Capon that are bound being a potential drug for decreasing nNOS activity.

15 29. The method of claim 28 wherein the test compound is contacted with at least one of the polypeptide and Capon prior to the step of contacting.

30. The method of claim 28 wherein one of the polypeptide and Capon is bound to a solid support.

15 31. The method of claim 28 wherein at least one of the polypeptide and Capon is radiolabeled.

32. The method of claim 28 wherein at least one of the polypeptide and Capon is a fusion protein.

20 33. The method of claim 28 wherein at least one of the polypeptide and Capon is a fusion protein that has a detectable enzyme activity.

34. A method of screening test compounds for the ability to decrease or augment nNOS activity, comprising the steps of:

25 (a) contacting a cell with a test compound, wherein the cell comprises:

i) a first fusion protein comprising (1) a DNA binding domain or a transcriptional activation domain, and (2) all or a portion of a mammalian Capon protein, wherein the portion consists of a contiguous sequence of amino acids selected from the amino acid sequence shown in SEQ ID NO:2 or 4, wherein the portion is capable of binding to nNOS;

30 ii) a second fusion protein comprising (1) a transcriptional activation domain or a DNA binding domain and (2) all or a portion of nNOS, wherein the portion comprises a PDZ domain, or a naturally occurring biologically active variant

5

thereof, wherein the interaction of the portion of the Capon protein with the portion of nNOS reconstitutes a sequence-specific transcriptional activating factor, wherein when the first fusion protein comprises a DNA binding domain the second fusion protein comprises a transcriptional activation domain and when the first fusion protein comprises a transcriptional activation domain the second fusion protein comprises a DNA binding domain; and

10

iii) a reporter gene comprising a DNA sequence to which the DNA binding domain of the first fusion protein specifically binds; and

(b) measuring the expression of the reporter gene, a test compound that increases the expression of the reporter gene being a potential drug for decreasing nNOS activity, and a test compound that decreases the expression of the reporter gene potential drug for augmenting nNOS activity

15

35. A method of screening for drugs with the ability to decrease or augment nNOS activity comprising the steps of:

20

(a) contacting a cell with a test compound, wherein the cell comprises:  
(i) a first expression vector comprising a subgenomic polynucleotide encoding at least the PDZ domain of nNOS or a naturally occurring biologically active variant thereof.

23

(b) measuring the amount of nitric oxide produced by the cell, a test compound that increases the amount of nitric oxide being a potential drug for augmenting nNOS activity, and a test compound that decreases the amount of nitric oxide being a potential drug for decreasing nNOS activity.

36. A method of diagnosing a neurological disease or propensity for a neurological disease, comprising:

determining number of glutamine repeats present in a Capon protein of a patient wherein a number greater than 6 indicates a neurologic disease or a propensity therefor.

37. A method of diagnosing a neurological disease or a propensity for a neurological disease, comprising:

determining number of CAG repeats in a *Capon* gene of a patient, wherein a number greater than 6 indicates a neurologic disease or a propensity therefor.

5 38. A cell comprising one or more recombinant constructs, comprising:

(i) a first expression vector comprising a subgenomic polynucleotide encoding at least the PDZ domain of nNOS or a naturally occurring biologically active variant thereof;

10 (ii) a second expression vector comprising a subgenomic polynucleotide encoding at least a portion of Capon or a naturally occurring biologically active variant thereof, wherein the portion of Capon is capable of binding to nNOS.

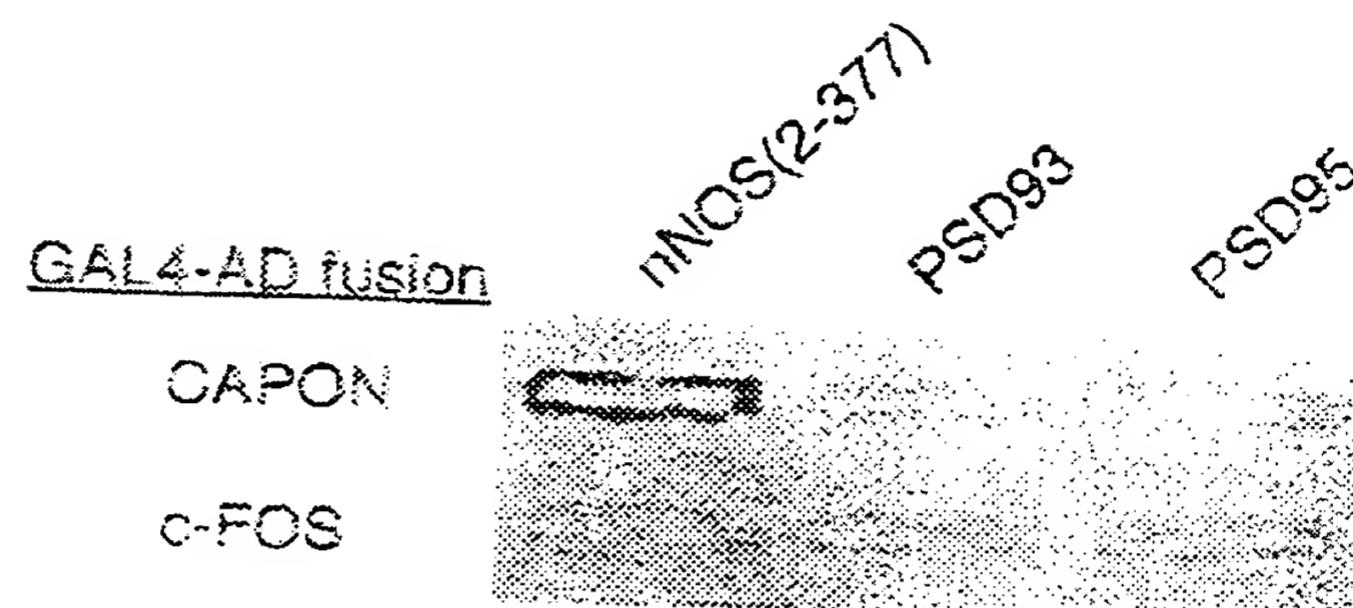
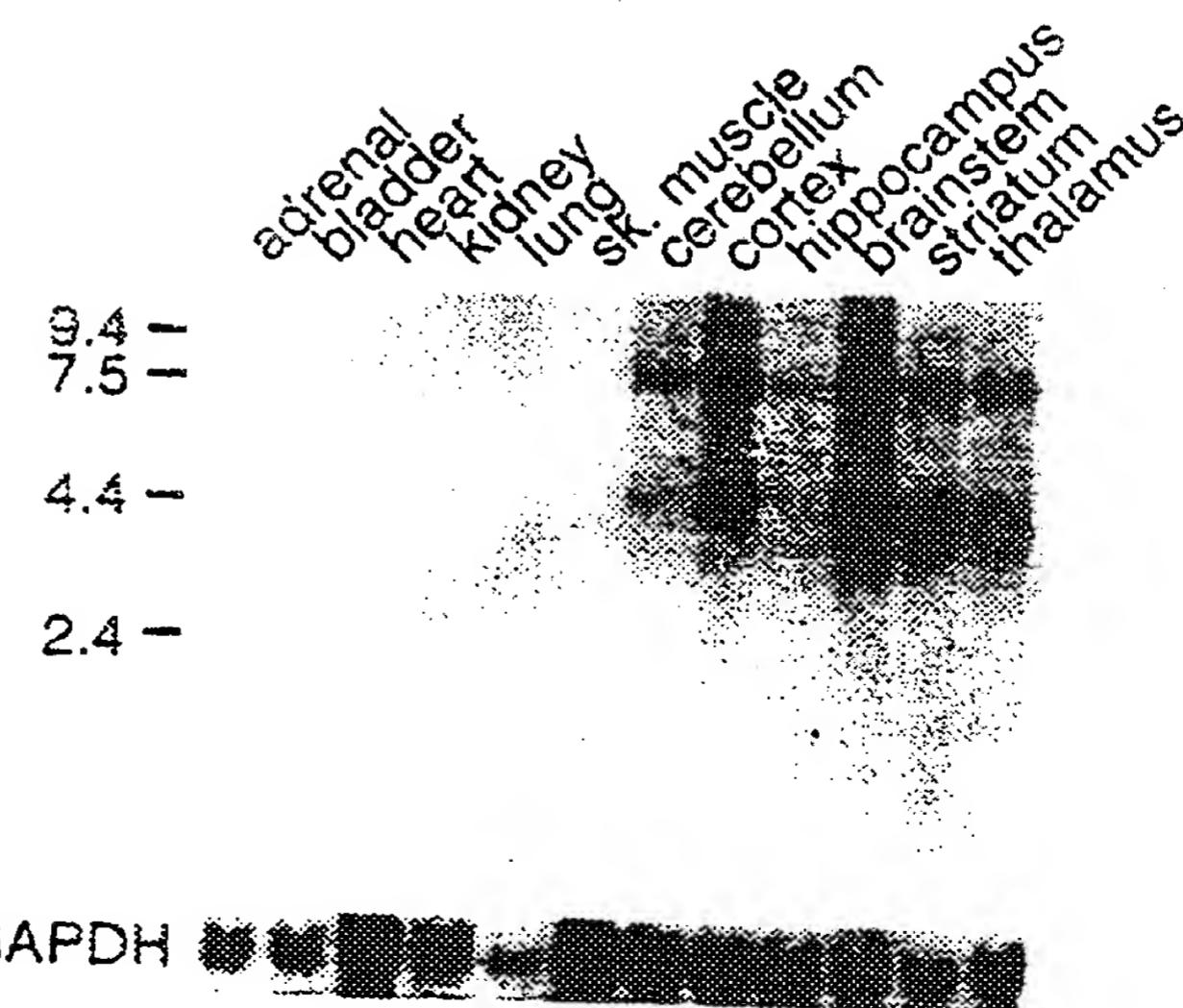
39. A cell comprising one or more recombinant constructs, comprising:

15 i) a nucleotide construct encoding a first fusion protein comprising (1) a DNA binding domain or a transcriptional activation domain, and (2) all or a portion of a mammalian Capon protein, wherein the portion consists of a contiguous sequence of amino acids selected from the amino acid sequence shown in SEQ ID NO:2 or 4, wherein the portion is capable of binding to nNOS;

20 ii) a nucleotide construct encoding a second fusion protein comprising (1) a transcriptional activation domain or a DNA binding domain and (2) all or a portion of nNOS, wherein the portion comprises a PDZ domain, or a naturally occurring biologically active variant thereof, wherein the interaction of the portion of the Capon protein with the portion of nNOS reconstitutes a sequence-specific transcriptional activating factor, wherein when the first fusion protein comprises a DNA binding domain the second fusion protein comprises a transcriptional activation domain and when the first fusion protein comprises a transcriptional activation domain the second fusion protein comprises a DNA binding domain; and

25 30 iii) a reporter gene comprising a DNA sequence to which the DNA binding domain of the first fusion protein specifically binds, wherein upon reconstitution of the sequence specific transactivating factor, expression of the reporter gene is increased.

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**FIG. 1A****FIG. 1C****SUBSTITUTE SHEET (RULE 26)**

**FIG. 1B**

1 MPSKTKYNLV DDGHDLRIPL HNEDAFQHGI SFEAKYVGSL  
 41 DVPRPNSRVE IVAAMRRIRY EFKAKNIKKK KVSIIMVSVDG  
 81 VKVILKKKK KKEWTWDESK MLVMDPPIYR IFYVSHDSQD  
 121 KIFSYIARD GASNIFRCNV FKSKKKSOAM RIVRTYGOAF  
 161 EYCHKLSLOH TQONADGOED GESERNSDGS GDPGROLTGA  
 201 ERVSTATEE TDIDAVEVPL PGNDILEFSR GVTDLDAIGK  
 241 DGGSHIDTV SPHPOEPHLA ASPRMLLPSS SSSKPPGLGT  
 281 GTPLSLTHOM OLLOOLLOO QOQTAVAVAO VHLLKDOLAA  
 321 EAARLEAQAA RVHQLLONK DMLOHISLLV KOVOELELKL  
 hCAPON EST  
 361 SGOSTMGSQD SLLEITFRSG ALPVLCESTT PKPEDLHSPL  
 hCAPON EST SGONAMGSQD SLLEITFRSG ALPVLCDPTT PKPEDLHSPP  
 401 LGAGLADFAH PGSPPLGRRD CLVKLECFRF LPAEDNOPMA  
 hCAPON EST LGAGLADFAH PAGSPPLGRRD CLVKLECFRF LPPEDTPPPA  
 441 OGEPLLGGL LIKFRESGIA SEYESNTDES EERDSWSQEE  
 hCAPON EST OGEAALLGGL LIKFRESGIA SEYESNTDES EERDSWSQEE  
 481 LPRLLNVLQR QELGDSLDDDE IAV  
 hCAPON EST LPRLLNVLQR QELGDGLDDDE IAV

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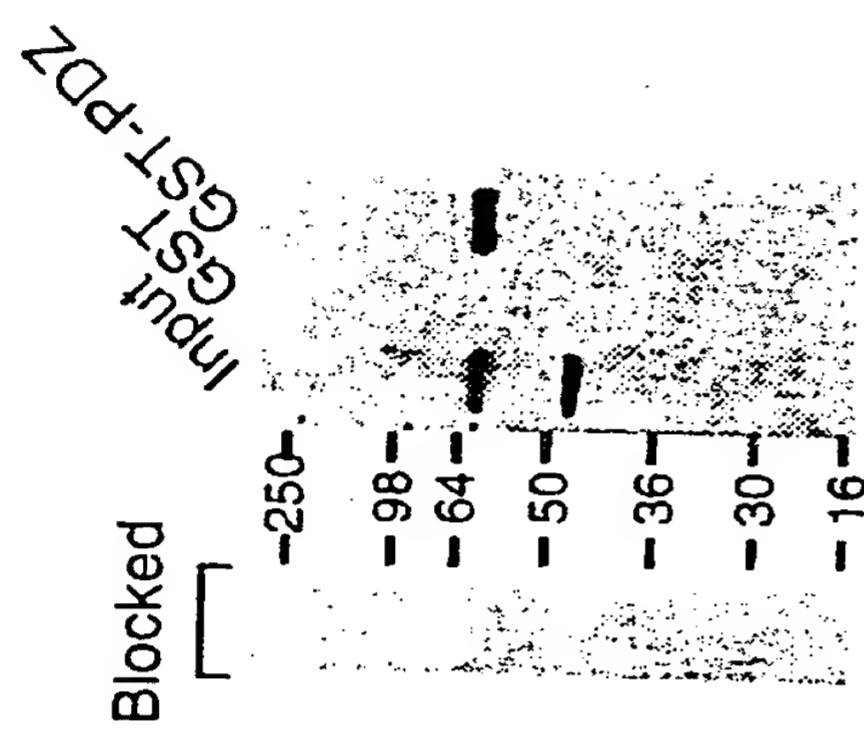


FIG. 2B

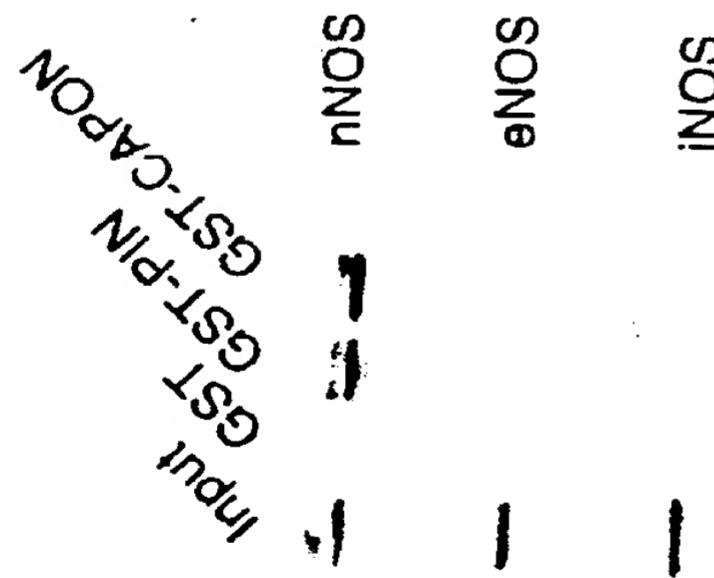


FIG. 2A

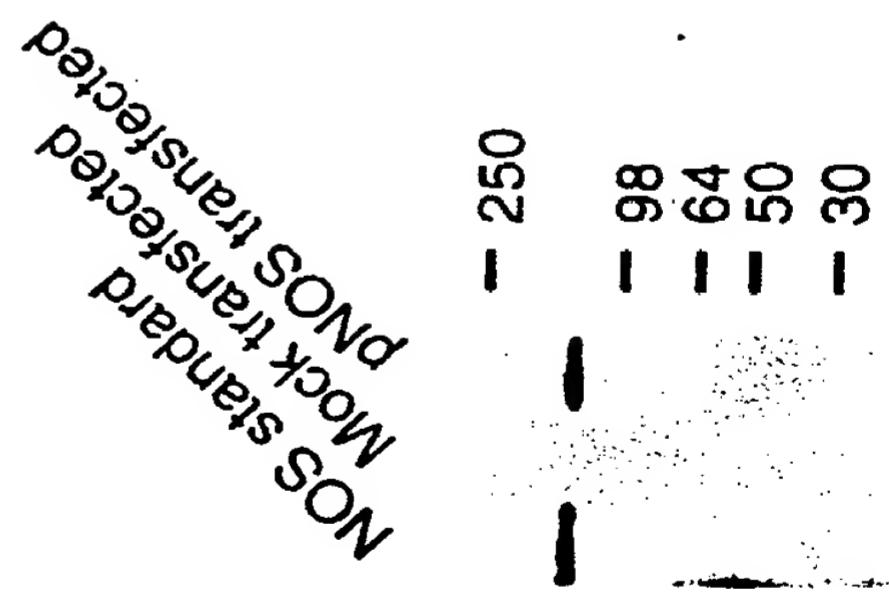


FIG. 2C

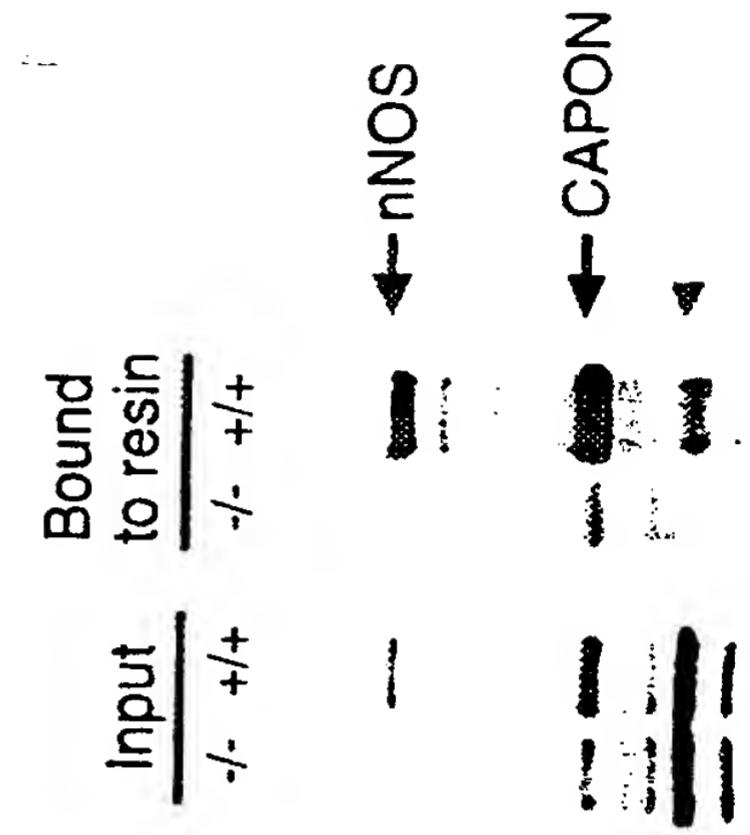
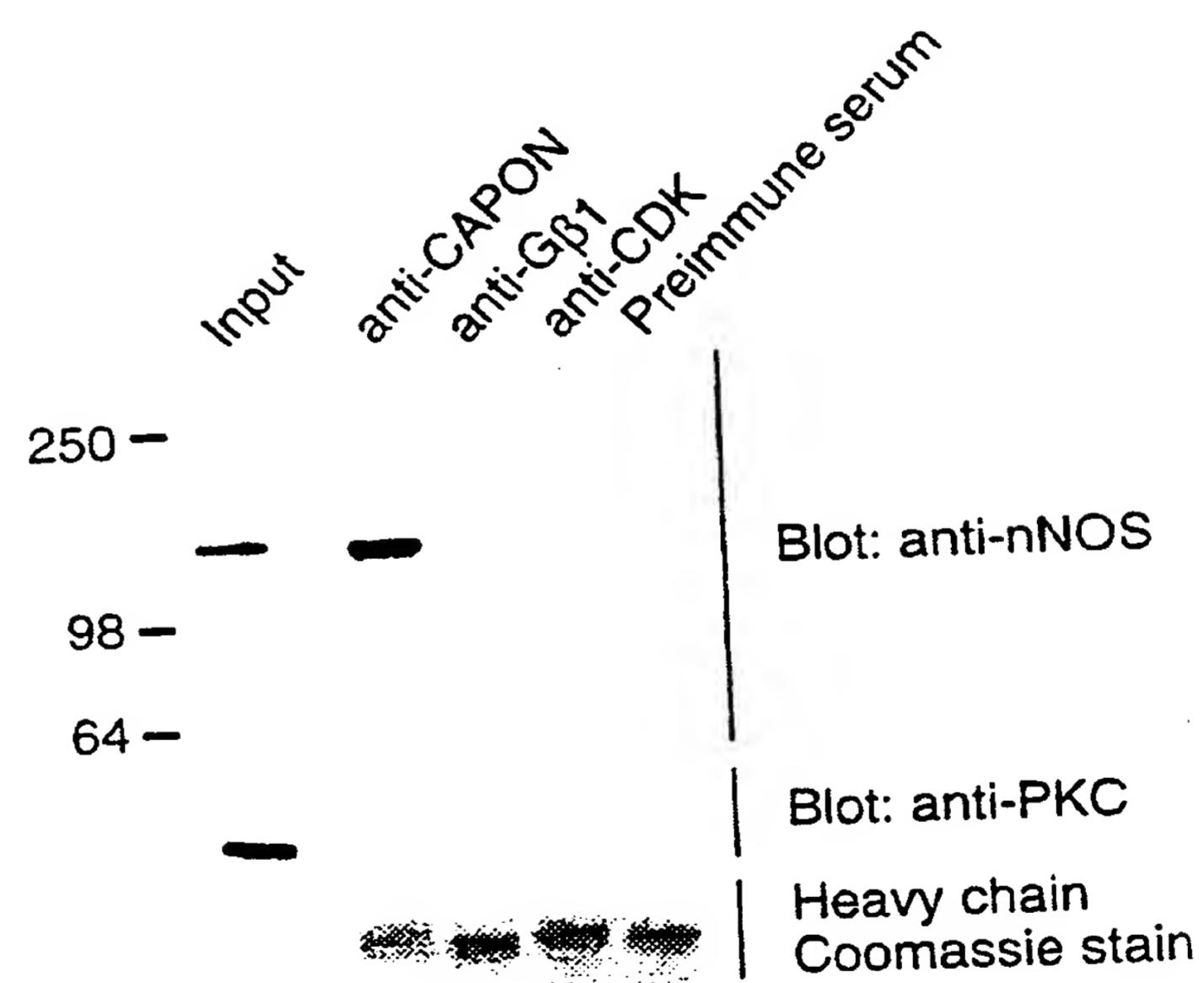


FIG. 2D

Probe: 32P-GST-CAPON

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**FIG. 2E**

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FIG. 3A

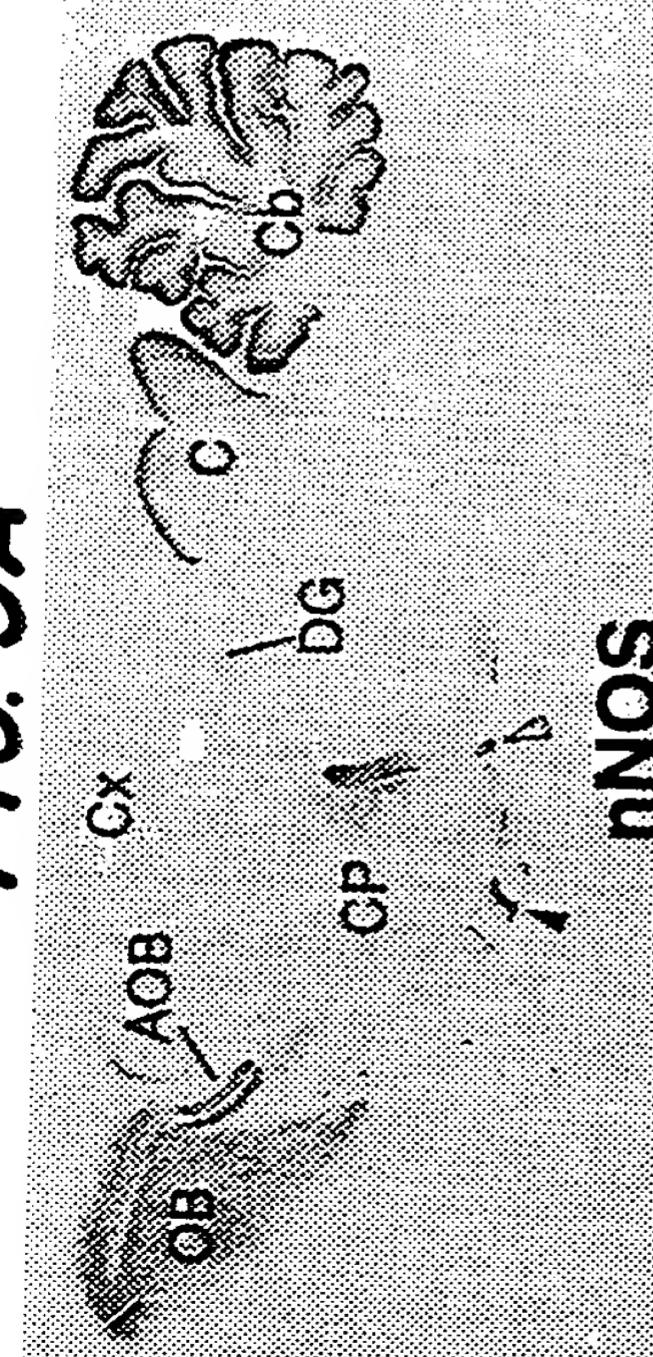
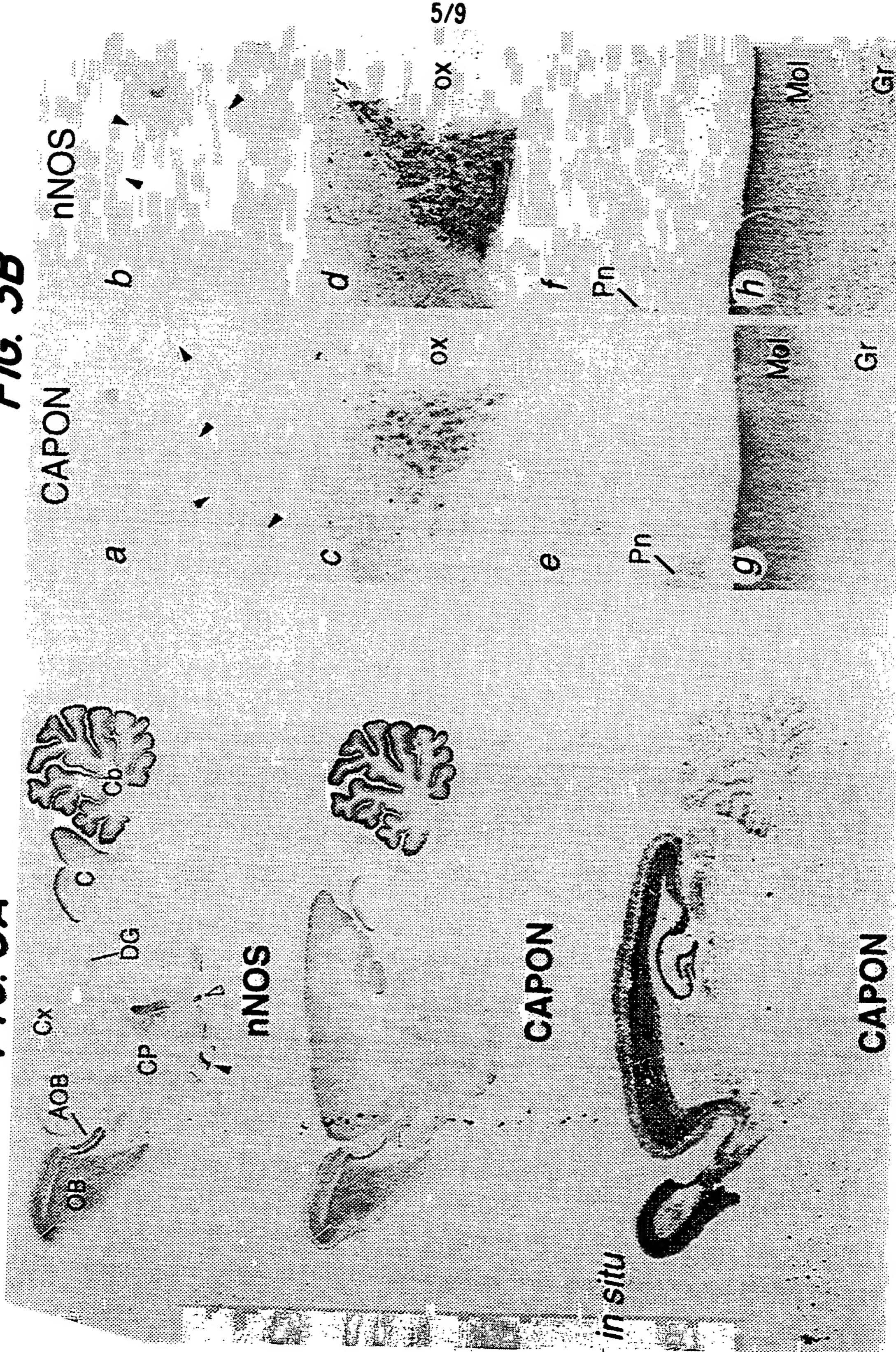


FIG. 3B



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FIG. 4A

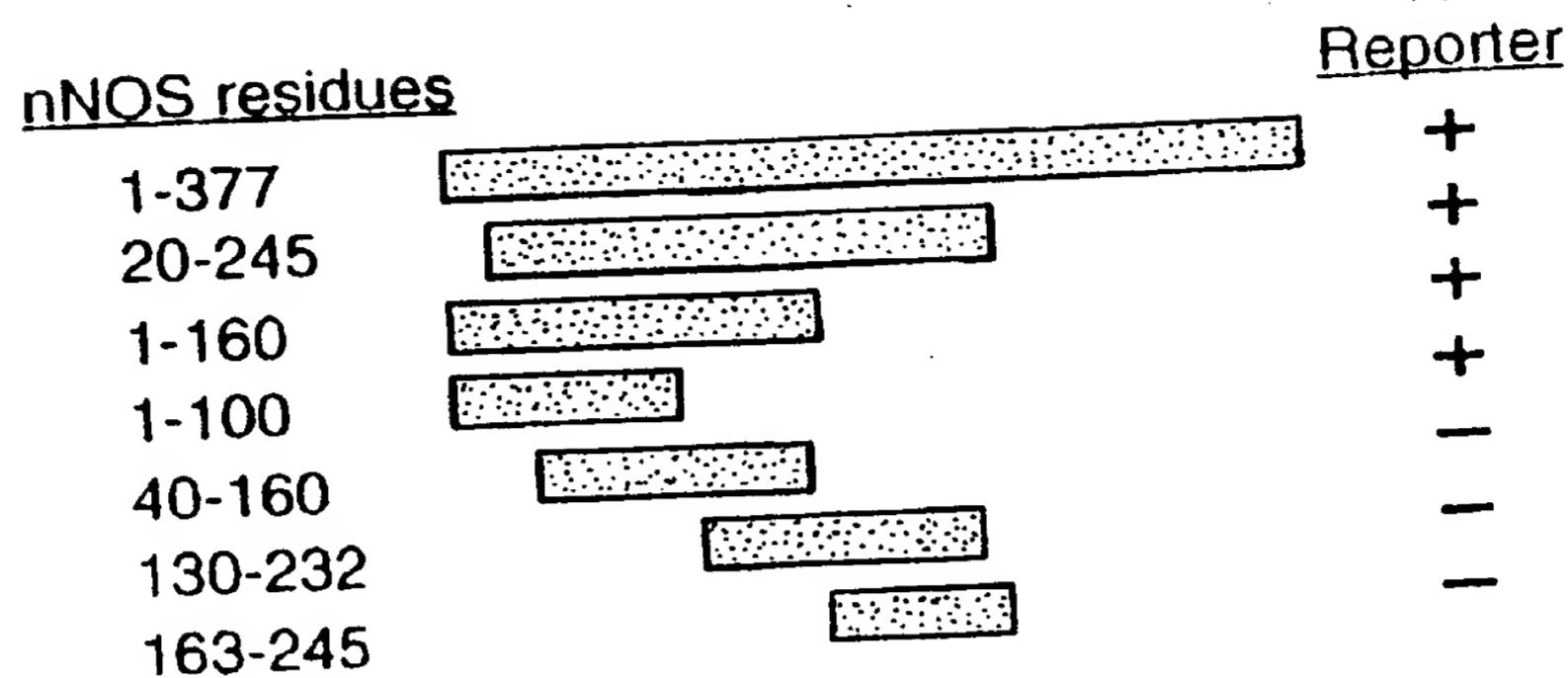


FIG. 4B

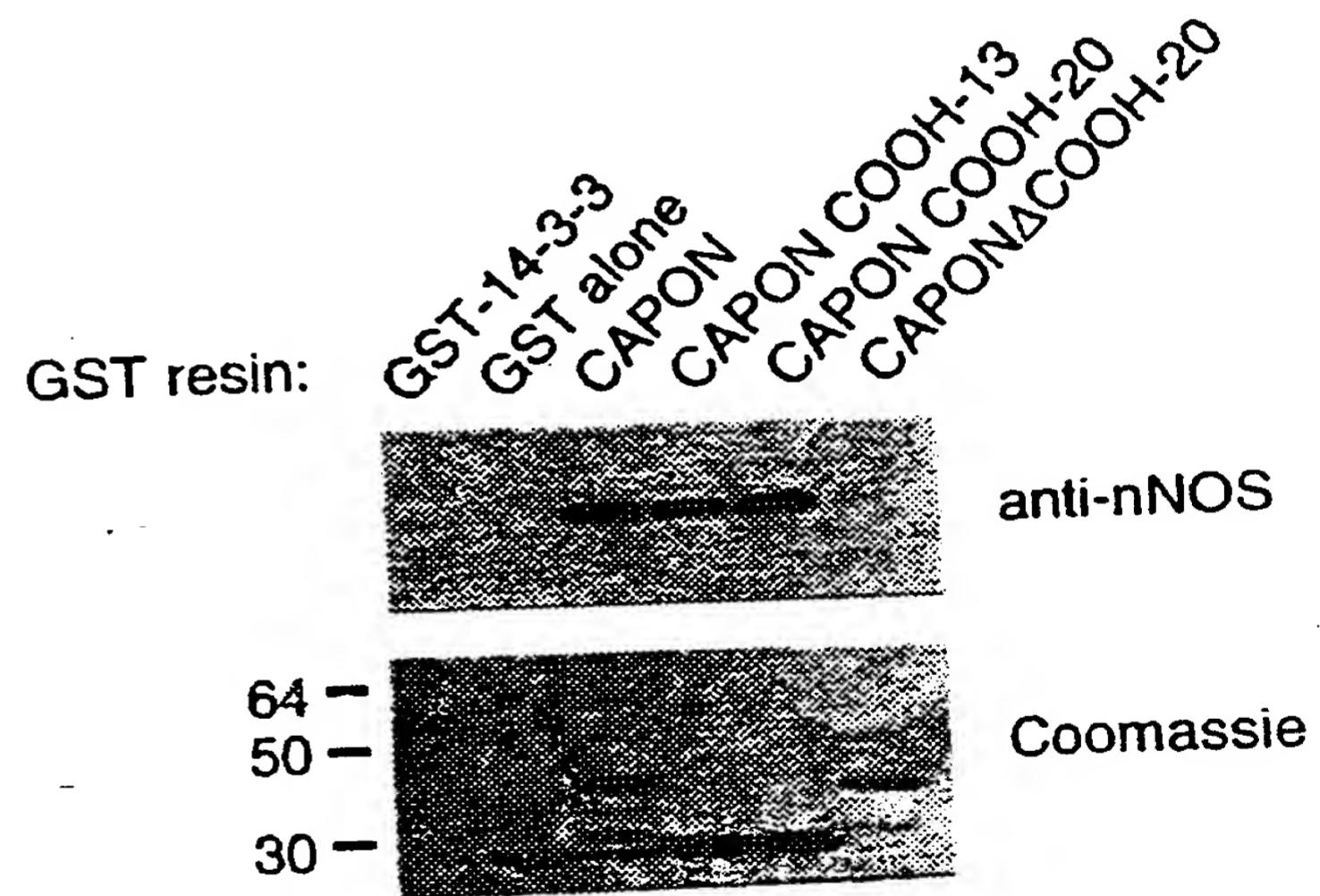
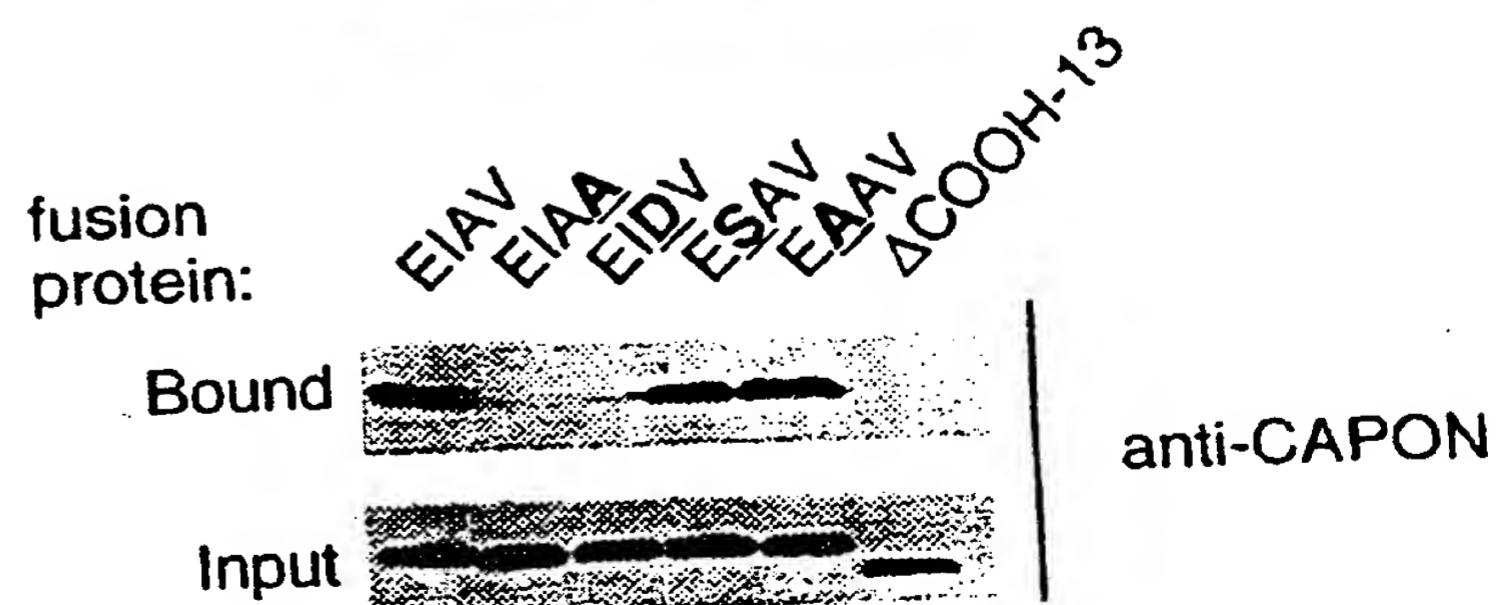
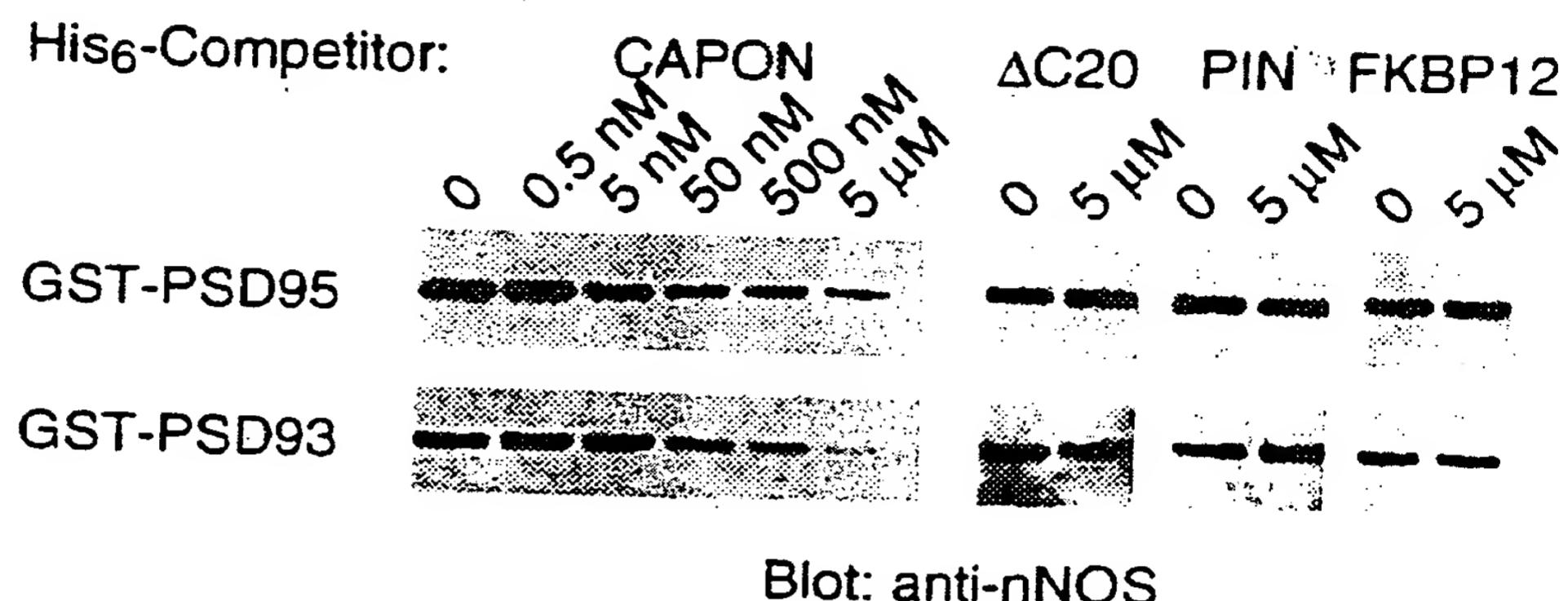
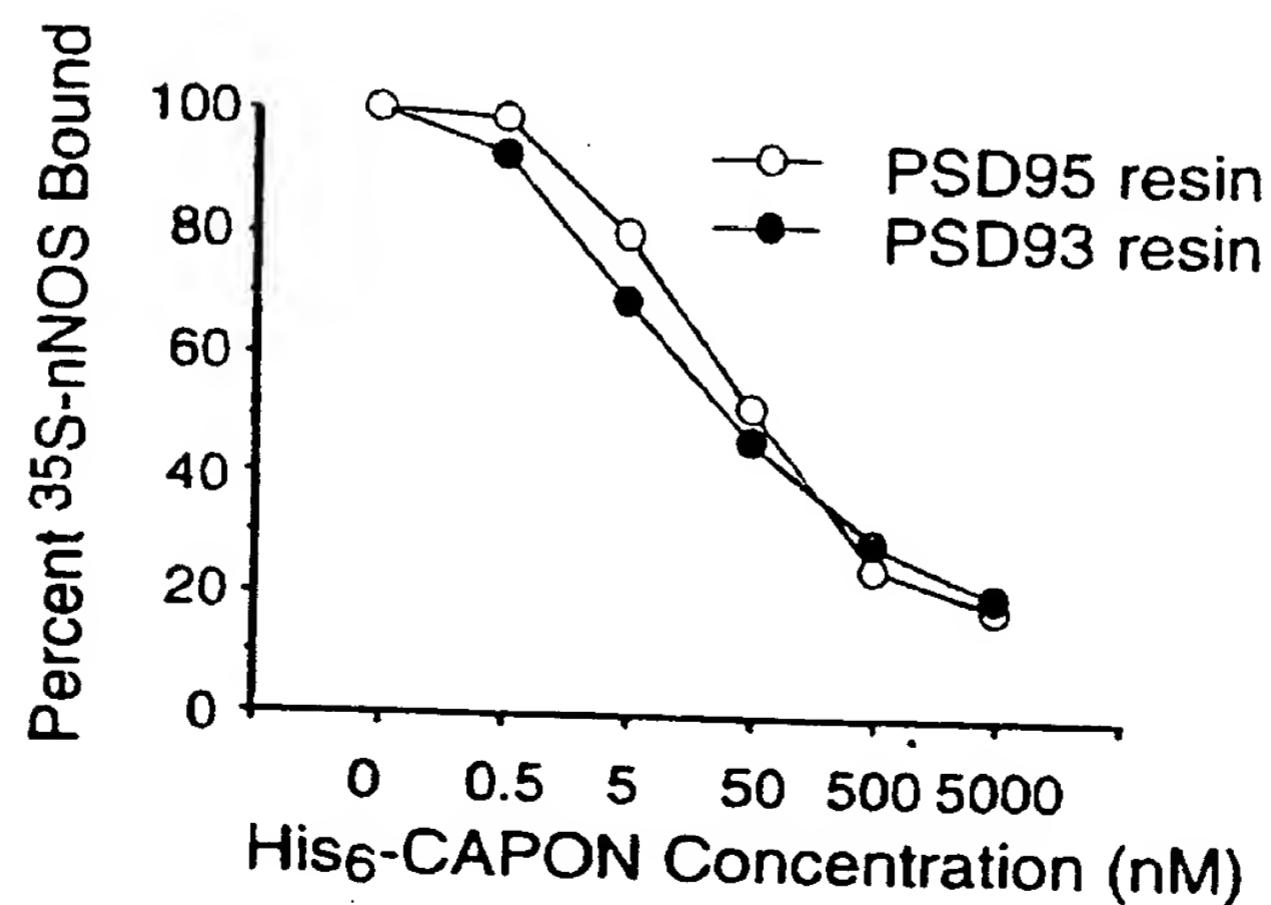


FIG. 4C



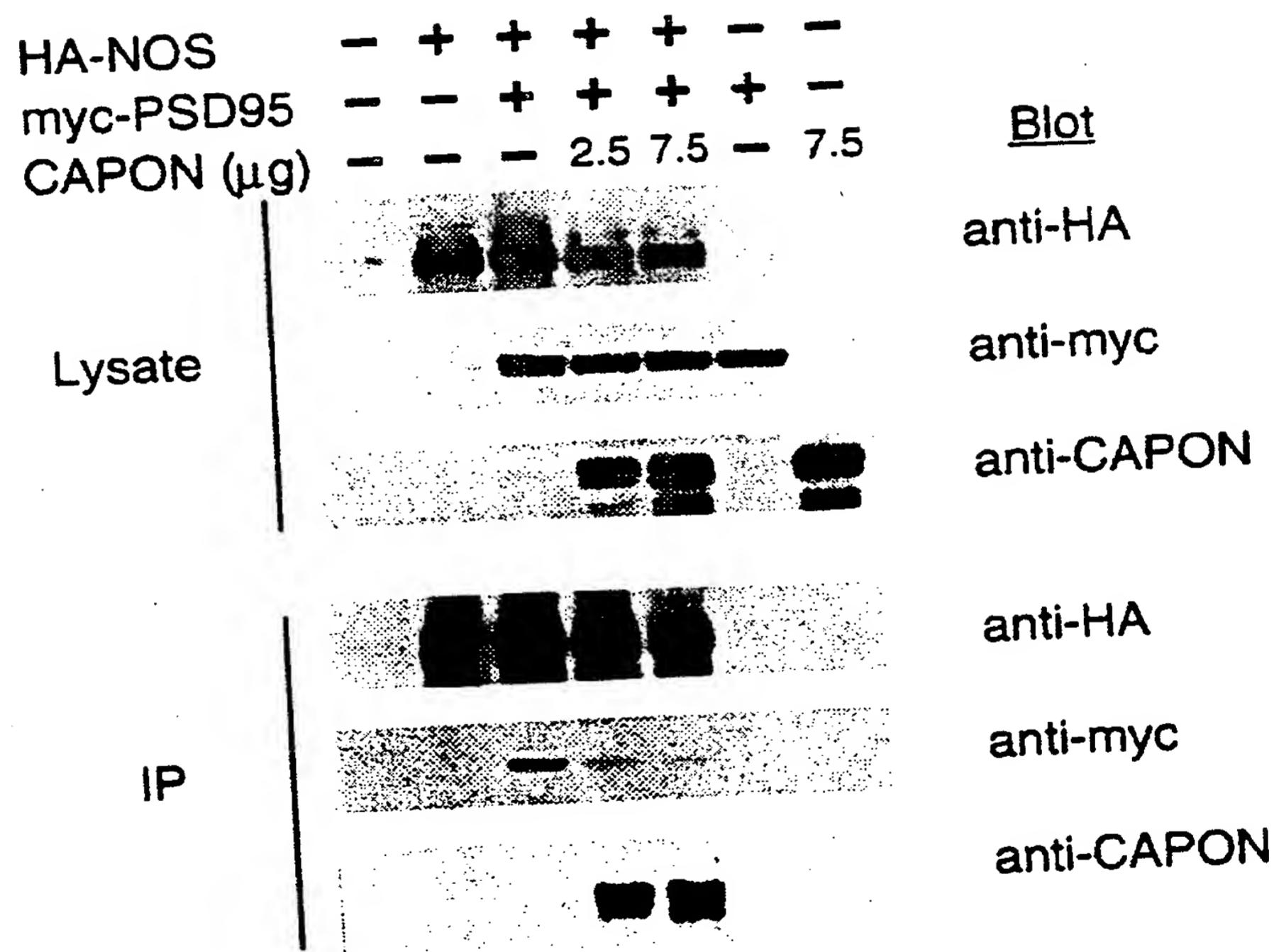
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**FIG. 5A****FIG. 5B**

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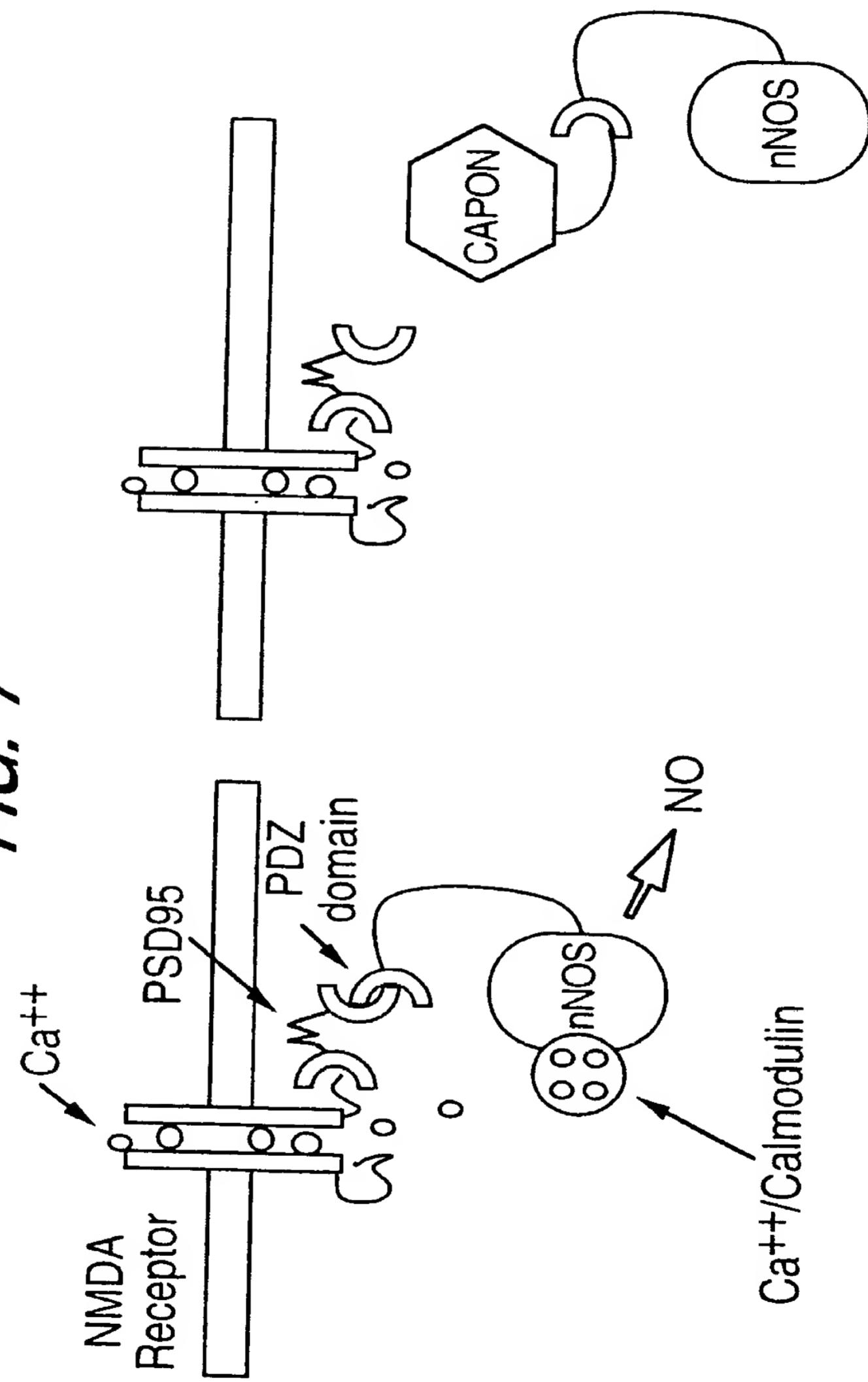
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**FIG. 6**

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FIG. 7



Model of PSD95/nNOS Regulation by CAPON

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION

(i) APPLICANT: Snyder, Solomon  
Jaffrey, Samie

5 (ii) TITLE OF THE INVENTION: CAPON, a protein that binds  
neuronal nitric oxide synthase

(iii) NUMBER OF SEQUENCES: 6

## (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Banner & Witcoff  
(B) STREET: 1001 G Street, NW  
(C) CITY: Washington  
(D) STATE: DC  
(E) COUNTRY: USA  
(F) ZIP: 20001

15 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette  
(B) COMPUTER: IBM Compatible  
(C) OPERATING SYSTEM: DOS  
(D) SOFTWARE: FastSEQ for Windows Version 2.0

20 (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE: 22-JAN-1998  
(C) CLASSIFICATION:

25 (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:

30 (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Kagan, Sarah A  
(B) REGISTRATION NUMBER: 32141  
(C) REFERENCE/DOCKET NUMBER: 01107.73424

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 202-508-9100  
(B) TELEFAX: 202-508-9299  
(C) TELEX:

35 (2) INFORMATION FOR SEQ ID NO:1:

5 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 2826 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATTCTGGCA CGAGCCGGGT CGTGCAGGCC GAGCTCGGGGA TCCGGCTCCC AGTCTAGCCC  
 60 CGCTTCGGGC CGTGCAGCCCT TTGCTCGGCG TCCGGCTCCG GGGCTCCGCG CCACCCGCTC  
 120 CCGCCTGCCCG GGCCGCCTGG CCGCCTCCCC GTAGTCAGAG CGCGGCCACC GAGCTGCTCG  
 180 CGCCAGCCGC ATCCGCGCCG CCCCTGCCGA TCGGCCCTCC GGAGGCACCG CTCCGGGTCC  
 240 CCCCCGCCAC TGCCTGGCAC CCAGGCTGCC CACCTCGCGA CCCGGGTCC CGCTGCCGCC  
 300 TCGCCCGGCC CCACTGTTCT CTCCACGGGG TCTCGCCAGC TCTTCTCGT CGCCGCCACC  
 360 GCCGCCCT TGGAGCAGCG GGTCCGCCGC GGGTCACCAT GCCCAGCAAA ACCAAGTACA  
 420 ACCTTGTGGA CGATGGGCAC GACTTACGGA TCCCTTGCA CAACGAGGAC GCCTTCCAGC  
 480 ACGGCATCTC TTTGAGGCC AAGTACGTGG GAAGCCTGGA TGTGCCAGA CCCAACAGCA  
 540 GGGTTGAGAT CGTGGCTGCC ATGCGCAGAA TCCGGTATGA GTTAAAGCC AAGAATATCA  
 600 AGAAGAAGAA AGTAAGCAGTC ATGGTCTCCG TGGACGGTGT CAAAGTGATT CTGAAGAAGA  
 660 AGAAAAAGAA AAAGGAGTGG ACGTGGGATG AGAGCAAGAT GCTGGTGATG CAGGACCCCTA  
 720 TCTACAGGAT CTTCTATGTC TCTCATGACT CCCAAGACTT GAAAATCTTC AGCTATATCG  
 780 CTCGAGATGG TGCCAGCAAT ATCTTCAGAT GCAATGTCTT TAAATCCAAG AAGAAGAGCC  
 840 AAGCTATGAG AATCGTACGG ACAGTGGGAC AGGCCTTGAG GGTCTGCCAC AAGCTGAGCC  
 900 TGCAGCACAC ACAGCAGAAT GCAGATGGCC AGGAAGATGG AGAGAGCGAG AGGAACAGCG  
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 1020 CAGAGGAGAC CGACATTGAC GCTGTGGAGG TCCCACCTCCC CGGGAAATGAC ATTCTAGAAT  
 1080 TCAGCCGAGG TGTGACTGAC CTGGATGCTA TTGGGAAGGA CGGAGGCTCC CACATAGACA  
 1140 CGACGGTCTC ACCCCATCCA CAGGAGCCCA TGCTGGCAGC CTCCCTCGC ATGCTGCTCC  
 1200 CTTCTTCTTC TTCTCGAAG CCACCGGGCT TGGGCAGTGG GACGCCCTG TCCACTCACC  
 1260 ACCAGATGCA GCTCCTCCAG CAGCTCCTCC AGCAGCAGCA GCAGCAGACA CAAGTGGCTG  
 1320

1380 TGGCTCAGGT TCACTTGCTG AAGGATCAGT TGGCTGCTGA GGCTGCGGCA CGGCTGGAGG  
 1440 CCCAGGCACG AGTGCACCAAG CTCCTGCTAC AGAACAAAGA CATGCTTCAG CACATCTCTC  
 5 1500 TGCTGGTTAA GCAGGTGCAG GAGCTGGAAC TGAAGCTGTC AGGACAGAGC ACCATGGGCT  
 1560 CCCAGGACAG CTTGCTGGAG ATCACCTTCC GTTCAGGTGC CCTGCCTGTG CTCTGTGAAT  
 10 1620 CCACCACTCC TAAGCCAGAG GACCTACACT CACCACTGCT GGGCGCTGGC TTGGCTGACT  
 1680 TTGCCCACCC AGTGGGCAGC CCCTTAGGTA GGCGTGACTG CTTGGTGAAG CTGGAGTGCT  
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 15 1800 ACGAAAGCGA GGAGCGTGAC TCGTGGTCGC AGGAAGAGCT GCCACGCCTG CTCAATGTCC  
 1860 TACAGCGGCA GGAGTTGGGT GACAGTTGG ATGATGAGAT CGCCGTGTAG GTGCAGGGCA  
 20 1920 AGGAGCTGGT GAAGGTGGCA GCATGATGCC AAGGGGGTCA AGTCTGCCTG TCCCCGGCTG  
 1980 GGGAAAGCCA GGGGAAAGCA CCGCTGAGAA AAACACCCAG GGCTGAGAGT GTAGGGTTTC  
 2040 2100 AGAAGAGGGT TGGGATTTG CTTTGGAAAGG TAAAGCAGGG AAGAAAATGG ATTCTAGAC  
 2160 ACAGGAATCA GCACCTGTAT TCTGCTAATG ACTGAATGGG ACGGAAGCAG GGCTTTCCAG  
 25 2220 AACCCAGGAC CTTGGGATGG GTCCGCCTTC AAGAATCACA GTTCTGGAAG GCCTGTTGCT  
 2280 CCCACCGTTA TAGTCAGGTT CTACTCAATC TGTCCGTGAT GTCTCAGTGG CCTACACTCT  
 2340 CCTGTCTCTG TGGTGCAGAT CATAAATGGA AGCCATTGAT ACCGTCTCAC GTACTTTGTT  
 30 2400 TTGGATATCA GGATGCTACA AGTTGCCTAA CCCTCCCTTA AGCTGTAGGA GAATTCCCTC  
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 240 ACTCTGCCAT TCAGTGAGCA TGGAGGTGAC AGCAGTCACA AGTCACAGTG AATAAACTAG  
 40 2520 GAATTCTACTC TAAGTGGGGT GTTGGATGTT GCTTCTGAGG AAGCTAGGAG TATGAATAGG  
 2580 ATTGAGGACC CTGCAGGGAG AGCCTGGGA GGGTTAGCCT AGGGGAGGGT TAGCCTAGAG  
 2640 45 AAGGGTTAGC CTAGGAGTGC TGATGACAGT TGTGGCAGCT CATGTAGGTG TGATTCTTCA  
 2700 GTTGGAAAC CATGCCCTT ACCCATCTCC TGCCTGCAAC CCAGCTCATA TAAACGAGGC  
 2760 TAAGAACTAT CATAATATCC CCTTTCTTG CCTCAGGGC TGTGCCTGCC TAATGAGTGC  
 50 2820 GGCCGC  
 2826

(2) INFORMATION FOR SEQ ID NO:2:

5

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 503 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

	Met	Pro	Ser	Lys	Thr	Lys	Tyr	Asn	Leu	Val	Asp	Asp	Gly	His	Asp	Leu
10	1				5				10					15		
	Arg	Ile	Pro	Leu	His	Asn	Glu	Asp	Ala	Phe	Gln	His	Gly	Ile	Ser	Phe
					20				25					30		
	Glu	Ala	Lys	Tyr	Val	Gly	Ser	Leu	Asp	Val	Pro	Arg	Pro	Asn	Ser	Arg
					35				40					45		
15	Val	Glu	Ile	Val	Ala	Ala	Met	Arg	Arg	Ile	Arg	Tyr	Glu	Phe	Lys	Ala
	50				55				55				60			
	Lys	Asn	Ile	Lys	Lys	Lys	Lys	Val	Ser	Ile	Met	Val	Ser	Val	Asp	Gly
	65			70				70			75				80	
20	Val	Lys	Val	Ile	Leu	Lys	Lys	Lys	Lys	Lys	Lys	Glu	Trp	Thr	Trp	
					85				90				95			
	Asp	Glu	Ser	Lys	Met	Leu	Val	Met	Gln	Asp	Pro	Ile	Tyr	Arg	Ile	Phe
					100				105				110			
	Tyr	Val	Ser	His	Asp	Ser	Gln	Asp	Leu	Lys	Ile	Phe	Ser	Tyr	Ile	Ala
					115				120				125			
25	Arg	Asp	Gly	Ala	Ser	Asn	Ile	Phe	Arg	Cys	Asn	Val	Phe	Lys	Ser	Lys
					130				135			140				
	Lys	Lys	Ser	Gln	Ala	Met	Arg	Ile	Val	Arg	Thr	Val	Gly	Gln	Ala	Phe
	145				150				155					160		
	Glu	Val	Cys	His	Lys	Leu	Ser	Leu	Gln	His	Thr	Gln	Gln	Asn	Ala	Asp
					165				170				175			
30	Gly	Gln	Glu	Asp	Gly	Glu	Ser	Glu	Arg	Asn	Ser	Asp	Gly	Ser	Gly	Asp
					180				185				190			
	Pro	Gly	Arg	Gln	Leu	Thr	Gly	Ala	Glu	Arg	Val	Ser	Thr	Ala	Thr	Ala
					195				200				205			
35	Glu	Glu	Thr	Asp	Ile	Asp	Ala	Val	Glu	Val	Pro	Leu	Pro	Gly	Asn	Asp
					210				215				220			
	Ile	Leu	Glu	Phe	Ser	Arg	Gly	Val	Thr	Asp	Leu	Asp	Ala	Ile	Gly	Lys
	225				230				235					240		
	Asp	Gly	Gly	Ser	His	Ile	Asp	Thr	Thr	Val	Ser	Pro	His	Pro	Gln	Glu
					245				250				255			
40	Pro	Met	Leu	Ala	Ala	Ser	Pro	Arg	Met	Leu	Leu	Pro	Ser	Ser	Ser	Ser
					260				265				270			
	Ser	Lys	Pro	Pro	Gly	Leu	Gly	Thr	Gly	Thr	Pro	Leu	Ser	Thr	His	His
					275				280				285			
45	Gln	Met	Gln	Leu	Leu	Gln	Gln	Leu	Leu	Gln	Gln	Gln	Gln	Gln	Gln	Thr
					290				295				300			
	Gln	Val	Ala	Val	Ala	Gln	Val	His	Leu	Leu	Lys	Asp	Gln	Leu	Ala	Ala
	305				310				315					320		
	Glu	Ala	Ala	Ala	Arg	Leu	Glu	Ala	Gln	Ala	Arg	Val	His	Gln	Leu	Leu
					325				330				335			
50	Leu	Gln	Asn	Lys	Asp	Met	Leu	Gln	His	Ile	Ser	Leu	Leu	Val	Lys	Gln
					340				345				350			

val Gln Glu Leu Glu Leu Lys Leu Ser Gly Gln Ser Thr Met Gly Ser  
 355 360 365  
 Gln Asp Ser Leu Leu Glu Ile Thr Phe Arg Ser Gly Ala Leu Pro Val  
 370 375 380  
 5 Leu Cys Glu Ser Thr Thr Pro Lys Pro Glu Asp Leu His Ser Pro Leu  
 385 390 395 400  
 Leu Gly Ala Gly Leu Ala Asp Phe Ala His Pro Val Gly Ser Pro Leu  
 405 410 415  
 Gly Arg Arg Asp Cys Leu Val Lys Leu Glu Cys Phe Arg Phe Leu Pro  
 10 420 425 430  
 Ala Glu Asp Asn Gln Pro Met Ala Gln Gly Glu Pro Leu Leu Gly Gly  
 435 440 445  
 Leu Glu Leu Ile Lys Phe Arg Glu Ser Gly Ile Ala Ser Glu Tyr Glu  
 450 455 460  
 15 Ser Asn Thr Asp Glu Ser Glu Glu Arg Asp Ser Trp Ser Gln Glu Glu  
 465 470 475 480  
 Leu Pro Arg Leu Leu Asn Val Leu Gln Arg Gln Glu Leu Gly Asp Ser  
 485 490 495  
 Leu Asp Asp Glu Ile Ala Val  
 20 500

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1504 base pairs
- (B) TYPE: nucleic acid
- 25 (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AAGCTTGGCA CGAGGTCAAG CAGGTGCAAG AGCTGGAACG GAAGCTGTCA GGACAGAACG  
 30 60  
 CCATGGGCTC CCAGGACAGC TTGCTGGAGA TCACCTTCCG CTCCGGAGCC CTGCCCGTGC  
 120  
 180 TCTGTGACCC CACGACCCCT AAGCCAGAGG ACCTGCATTC GCCGCCGCTG GGCGCGGGCT  
 35 240 TGGCTGACTT TGCCCACCCCT GCGGGCAGCC CCTTAGGTAG GCGCGACTGC TTGGTGAAGC  
 300  
 40 360 TGGAGTGCTT TCGCTTCCTT CCGCCCGAGG ACACCCCGCC CCCAGCGCAG GGCGAGGCGC  
 420  
 480  
 45 540 TCCCTGGCGG TCTGGAGCTC ATCAAGTTCC GAGAGTCAGG CATCGCCTCG GAGTACGAGT  
 600  
 CCAACACCGA CGAGAGCGAG GAGCGCGACT CGTGGTCCCA GGAGGGAGCTG CCGCGCCTGC  
 TGAATGTCCT GCAGAGGCAG GAACTGGGCG ACGGCCTGGA TGATGAGATC GCCGTGTAGG  
 TGCCGAGGGC GAGGAGATGG AGGCGCGGGC GTGGCTGGAG GGGCCGTGTC TGGCTGCTGC  
 CCGGGTAGGG GATGCCAGT GAATGTGCAC TGCCGAGGAG AATGCCAGCC AGGGCCCCGGG

660 AGAGTGTGAG GTTCAGGAA AGTATTGAGA TTCTGCTTG GAGGGTAAAG TGGGAAAGAA  
 660  
 670 ATCGGATTCC CAGAGGTGAA TCAGCTCCTC TCCTACTTGT GACTAGAGGG TGGTGGAGGT  
 670  
 680 AAGGCCTTCC AGAGCCCAG GCTTCAGGAG AGGGTCTCTC TCCAGGACTG CCAGGCTGCT  
 680  
 690 GGAGGACCTG CCCCTACCTG CTGCATCGTC AGGCTCCCAC GCTTGTCGG TGATGCC  
 690  
 700 CTACCCCCCTC ACTCTCCCCG TCTCCATGGT CCCGACCAGG AAGGGAAGCC ATCGGTACCT  
 700  
 710 TCTCAGGTAC TTTGTTTCTG GATATCACGA TGCTGCGAGT TGCTAACCC TCCCCCTACC  
 710  
 720 TTTATGAGAG GAATTCCCTTC TCCAGGCCCT TGCTGAGATT GTAGAGATTG AGTGCTCTGG  
 720  
 730 1020 ACCGCAGAAAG CCAGGCTAGT CCTTGTAGGG TGAGCATGGA ATTGGAATGT GTCACAGTGG  
 730  
 740 1140 ATAAGCTTT AGAGGAACAG AATCCAAACA TTTTCTCCAG CCGGACATTG AATGTTGCTA  
 740  
 750 1200 CAAAGGGAGC CTTGAAGCTT TAACATGGTT CAGGCCCTTG GTGTGAGAGC CCAGGGGGAG  
 750  
 760 1260 GACAGCTTGT CTGCTGCTCC AAATCACTTA GATCTGATTG CTGTTTGAA AGTCCTGCC  
 760  
 770 1320 TGCCTTCCTC CTGCCTGTAG CCCAGCCCAT CTAATGGAA GCTGGAAATT GCCCCTCACC  
 770  
 780 1380 TCCCCTGTGT CCTGTCCAGC TGAAGCTTT GCAGCACTT ACCTCTCTGA AAGCCCCAGA  
 780  
 790 1440 GGACCAGAGC CCCCAGCCTT ACCTCTCAAC CTGTCCCCTC CACTGGCAG TGGTGGTCAG  
 790  
 800 1500 TTTTACTGC AAAAAAAA AAAAAGAAAA AAGAGAAAA AAAAAAAA ATTCCCTGCGG  
 800  
 810 1504 CCGC  
 810

## (2) INFORMATION FOR SEQ ID NO:4:

35 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 153 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: None

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

45 Lys Gln Val Gln Glu Leu Glu Leu Lys Leu Ser Gly Gln Asn Ala Met  
 1 5 10 15  
 Gly Ser Gln Asp Ser Leu Leu Glu Ile Thr Phe Arg Ser Gly Ala Leu  
 20 25 30  
 Pro Val Leu Cys Asp Pro Thr Thr Pro Lys Pro Glu Asp Leu His Ser  
 35 40 45  
 Pro Pro Leu Gly Ala Gly Leu Ala Asp Phe Ala His Pro Ala Gly Ser  
 50 55 60  
 Pro Leu Gly Arg Arg Asp Cys Leu Val Lys Leu Glu Cys Phe Arg Phe

65	70	75	80
	Leu Pro Pro Glu Asp Thr Pro Pro Pro Ala Gln Gly Glu Ala Leu Leu		
	85	90	95
5	Gly Gly Leu Glu Leu Ile Lys Phe Arg Glu Ser Gly Ile Ala Ser Glu		
	100	105	110
	Tyr Glu Ser Asn Thr Asp Glu Ser Glu Glu Arg Asp Ser Trp Ser Gln		
	115	120	125
10	Glu Glu Leu Pro Arg Leu Leu Asn Val Leu Gln Arg Gln Glu Leu Gly		
	130	135	140
	Asp Gly Leu Asp Asp Glu Ile Ala Val		
	145	150	

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1430 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: None

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

20	Met Glu Glu Asn Thr Phe Gly Val Gln Gln Ile Gln Pro Asn Val Ile		
	1	5	10
	Ser Val Arg Leu Phe Lys Arg Lys Val Gly Gly Leu Gly Phe Leu Val		
	20	25	30
25	Lys Glu Arg Val Ser Lys Pro Pro Val Ile Ile Ser Asp Leu Ile Arg		
	35	40	45
	Gly Gly Ala Ala Glu Gln Ser Gly Leu Ile Gln Ala Gly Asp Ile Ile		
	50	55	60
30	Leu Ala Val Asn Asp Arg Pro Leu Val Asp Leu Ser Tyr Asp Ser Ala		
	65	70	75
	Leu Glu Val Leu Arg Gly Ile Ala Ser Glu Thr His Val Val Leu Ile		
	85	90	95
35	Leu Arg Gly Pro Glu Gly Phe Thr Thr His Leu Glu Thr Thr Phe Thr		
	100	105	110
	Gly Asp Gly Thr Pro Lys Thr Ile Arg Val Thr Gln Pro Leu Gly Pro		
	115	120	125
40	Pro Thr Lys Ala Val Asp Leu Ser His Gln Pro Ser Ala Ser Lys Asp		
	130	135	140
	Gln Ser Leu Ala Val Asp Arg Val Thr Gly Leu Gly Asn Gly Pro Gln		
	145	150	155
45	160	165	170
	His Ala Gln Gly His Gly Gln Gly Ala Gly Ser Val Ser Gln Ala Asn		
	175		
	Gly Val Ala Ile Asp Pro Thr Met Lys Ser Thr Lys Ala Asn Leu Gln		
	180	185	190
	Asp Ile Gly Glu His Asp Glu Leu Leu Lys Glu Ile Glu Pro Val Leu		
	195	200	205
	Ser Ile Leu Asn Ser Gly Ser Lys Ala Thr Asn Arg Gly Gly Pro Ala		
	210	215	220
	Lys Ala Glu Met Lys Asp Thr Gly Ile Gln Val Asp Arg Asp Leu Asp		
	225	230	235
			240

	Gly Lys Ser His Lys Ala Pro Pro Leu Gly Gly Asp Asn Asp Arg Val		
	245	250	255
	Phe Asn Asp Leu Trp Gly Lys Asp Asn Val Pro Val Ile Leu Asn Asn		
5	260	265	270
	Pro Tyr Ser Glu Lys Glu Gln Ser Pro Thr Ser Gly Lys Gln Ser Pro		
	275	280	285
	Thr Lys Asn Gly Ser Pro Ser Arg Cys Pro Arg Phe Leu Lys Val Lys		
	290	295	300
10	Asn Trp Glu Thr Asp Val Val Leu Thr Asp Thr Leu His Leu Lys Ser		
	305	310	315
	Thr Leu Glu Thr Gly Cys Thr Glu His Ile Cys Met Gly Ser Ile Met		
	325	330	335
	Leu Pro Ser Gln His Thr Arg Lys Pro Glu Asp Val Arg Thr Lys Asp		
15	340	345	350
	Gln Leu Phe Pro Leu Ala Lys Glu Phe Leu Asp Gln Tyr Tyr Ser Ser		
	355	360	365
	Ile Lys Arg Phe Gly Ser Lys Ala His Met Asp Arg Leu Glu Glu Val		
	370	375	380
20	Asn Lys Glu Ile Glu Ser Thr Ser Thr Tyr Gln Leu Lys Asp Thr Glu		
	385	390	395
	Leu Ile Tyr Gly Ala Lys His Ala Trp Arg Asn Ala Ser Arg Cys Val		
	405	410	415
	Gly Arg Ile Gln Trp Ser Lys Leu Gln Val Phe Asp Ala Arg Asp Cys		
25	420	425	430
	Thr Thr Ala His Gly Met Phe Asn Tyr Ile Cys Asn His Val Lys Tyr		
	435	440	445
	Ala Thr Asn Lys Gly Asn Leu Arg Ser Ala Ile Thr Ile Phe Pro Gln		
	450	455	460
30	Arg Thr Asp Gly Lys His Asp Phe Arg Val Trp Asn Ser Gln Leu Ile		
	465	470	475
	Arg Tyr Ala Gly Tyr Lys Gln Pro Asp Gly Ser Thr Leu Gly Asp Pro		
	485	490	495
	Ala Asn Val Gln Phe Thr Glu Ile Cys Ile Gln Gln Gly Trp Lys Ala		
35	500	505	510
	Pro Arg Gly Arg Phe Asp Val Leu Pro Leu Leu Leu Gln Ala Asn Gly		
	515	520	525
	Asn Asp Pro Glu Leu Phe Gln Ile Pro Pro Glu Leu Val Leu Glu Val		
	530	535	540
40	Pro Ile Arg His Pro Lys Phe Asp Trp Phe Lys Asp Leu Gly Leu Lys		
	545	550	555
	Trp Tyr Gly Leu Pro Ala Val Ser Asn Met Leu Leu Glu Ile Gly Gly		
	565	570	575
	Leu Glu Phe Ser Ala Cys Pro Phe Ser Gly Trp Tyr Met Gly Thr Glu		
	580	585	590
45	Ile Gly Val Arg Asp Tyr Cys Asp Asn Ser Arg Tyr Asn Ile Leu Glu		
	595	600	605
	Glu Val Ala Lys Lys Met Asp Leu Asp Met Arg Lys Thr Ser Ser Leu		
	610	615	620
50	Trp Lys Asp Gln Ala Leu Val Glu Ile Asn Ile Ala Val Leu Tyr Ser		
	625	630	635
	Phe Gln Ser Asp Lys Val Thr Ile Val Asp His His Ser Ala Thr Glu		
	645	650	655
	Ser Phe Ile Lys His Met Glu Asn Glu Tyr Arg Cys Arg Gly Gly Cys		
	660	665	670

Pro Ala Asp Trp Val Trp Ile Val Pro Pro Met Ser Gly Ser Ile Thr  
 675 680 685  
 Pro Val Phe His Gln Glu Met Leu Asn Tyr Arg Leu Thr Pro Ser Phe  
 690 695 700  
 5 Glu Tyr Gln Pro Asp Pro Trp Asn Thr His Val Trp Lys Gly Thr Asn  
 705 710 715 720  
 Gly Thr Pro Thr Lys Arg Arg Ala Ile Gly Phe Lys Lys Leu Ala Glu  
 725 730 735  
 Ala Val Lys Phe Ser Ala Lys Leu Met Gly Gln Ala Met Ala Lys Arg  
 740 745 750  
 10 Val Lys Ala Thr Ile Leu Tyr Ala Thr Glu Thr Gly Lys Ser Gln Ala  
 755 760 765  
 Tyr Ala Lys Thr Leu Cys Glu Ile Phe Lys His Ala Phe Asp Ala Lys  
 770 775 780  
 15 Ala Met Ser Met Glu Glu Tyr Asp Ile Val His Leu Glu His Glu Ala  
 785 790 795 800  
 Leu Val Leu Val Val Thr Ser Thr Phe Gly Asn Gly Asp Pro Pro Glu  
 805 810 815  
 Asn Gly Glu Lys Phe Gly Cys Ala Leu Met Glu Met Arg His Pro Asn  
 820 825 830  
 20 Ser Val Gln Glu Glu Arg Lys Ser Tyr Lys Val Arg Phe Asn Ser Val  
 835 840 845  
 Ser Ser Tyr Ser Asp Ser Arg Lys Ser Ser Gly Asp Gly Pro Asp Leu  
 850 855 860  
 25 Arg Asp Asn Phe Glu Ser Thr Gly Pro Leu Ala Asn Val Arg Phe Ser  
 865 870 875 880  
 Val Phe Gly Leu Gly Ser Arg Ala Tyr Pro His Phe Cys Ala Phe Gly  
 885 890 895  
 His Ala Val Asp Thr Leu Leu Glu Leu Gly Gly Glu Arg Ile Leu  
 900 905 910  
 30 Lys Met Arg Glu Gly Asp Glu Leu Cys Gly Gln Glu Ala Phe Arg  
 915 920 925  
 Thr Trp Ala Lys Lys Val Phe Lys Ala Ala Cys Asp Val Phe Cys Val  
 930 935 940  
 35 Gly Asp Asp Val Asn Ile Glu Lys Pro Asn Asn Ser Leu Ile Ser Asn  
 945 950 955 960  
 Asp Arg Ser Trp Lys Arg Asn Lys Phe Arg Leu Thr Tyr Val Ala Glu  
 965 970 975  
 Ala Pro Asp Leu Thr Gln Gly Leu Ser Asn Val His Lys Lys Arg Val  
 980 985 990  
 40 Ser Ala Ala Arg Leu Leu Ser Arg Gln Asn Leu Gln Ser Pro Lys Phe  
 995 1000 1005  
 Ser Arg Ser Thr Ile Phe Val Arg Leu His Thr Asn Gly Asn Gln Glu  
 1010 1015 1020  
 45 Leu Gln Tyr Gln Pro Gly Asp His Leu Gly Val Phe Pro Gly Asn His  
 1025 1030 1035 1040  
 Glu Asp Leu Val Asn Ala Leu Ile Glu Arg Leu Glu Asp Ala Pro Pro  
 1045 1050 1055  
 Ala Asn His Val Val Lys Val Glu Met Leu Glu Arg Asn Thr Ala  
 1060 1065 1070  
 50 Leu Gly Val Ile Ser Asn Trp Lys Asp Glu Ser Arg Leu Pro Pro Cys  
 1075 1080 1085  
 Thr Ile Phe Gln Ala Phe Lys Tyr Tyr Leu Asp Ile Thr Thr Pro Pro  
 1090 1095 1100

Thr Pro Leu Gln Leu Gln Gln Phe Ala Ser Leu Ala Thr Asn Glu Lys  
 105 1110 1115 1120  
 Glu Lys Gln Arg Leu Leu Val Leu Ser Lys Gly Leu Gln Glu Tyr Glu  
 1125 1130 1135  
 5 Glu Trp Lys Trp Gly Lys Asn Pro Thr Met Val Glu Val Leu Glu Glu  
 1140 1145 1150  
 Phe Pro Ser Ile Gln Met Pro Ala Thr Leu Leu Leu Thr Gln Leu Ser  
 1155 1160 1165  
 Leu Leu Gln Pro Arg Tyr Tyr Ser Ile Ser Ser Ser Pro Asp Met Tyr  
 1170 1175 1180  
 10 Pro Asp Glu Val His Leu Thr Val Ala Ile Val Ser Tyr His Thr Arg  
 185 1190 1195 1200  
 Asp Gly Glu Gly Pro Val His His Gly Val Cys Ser Ser Trp Leu Asn  
 1205 1210 1215  
 15 Arg Ile Gln Ala Asp Asp Val Val Pro Cys Phe Val Arg Gly Ala Pro  
 1220 1225 1230  
 Ser Phe His Leu Pro Arg Asn Pro Gln Val Pro Cys Ile Leu Val Gly  
 1235 1240 1245  
 Pro Gly Thr Gly Ile Ala Pro Phe Arg Ser Phe Trp Gln Gln Arg Gln  
 1250 1255 1260  
 20 Phe Asp Ile Gln His Lys Gly Met Asn Pro Cys Pro Met Val Leu Val  
 265 1270 1275 1280  
 Phe Gly Cys Arg Gln Ser Lys Ile Asp His Ile Tyr Arg Glu Glu Thr  
 1285 1290 1295  
 25 Leu Gln Ala Lys Asn Lys Gly Val Phe Arg Glu Leu Tyr Thr Ala Tyr  
 1300 1305 1310  
 Ser Arg Glu Pro Asp Arg Pro Lys Lys Tyr Val Gln Asp Val Leu Gln  
 1315 1320 1325  
 Glu Gln Leu Ala Glu Ser Val Tyr Arg Ala Leu Lys Glu Gln Gly Gly  
 1330 1335 1340  
 30 His Ile Tyr Val Cys Gly Asp Val Thr Met Ala Ala Asp Val Leu Lys  
 345 1350 1355 1360  
 Ala Ile Gln Arg Ile Met Thr Gln Gln Gly Lys Leu Ser Glu Glu Asp  
 1365 1370 1375  
 35 Ala Gly Val Phe Ile Ser Arg Leu Arg Asp Asp Asn Arg Tyr His Glu  
 1380 1385 1390  
 Asp Ile Phe Gly Val Thr Leu Arg Thr Tyr Glu Val Thr Asn Arg Leu  
 1395 1400 1405  
 40 Arg Ser Glu Ser Ile Ala Phe Ile Glu Glu Ser Lys Lys Asp Ala Asp  
 1410 1415 1420  
 Glu Val Phe Ser Ser Pro  
 425 1430

(2) INFORMATION FOR SEO ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

**(A) LENGTH: 1554 amino acids**

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(B) TOPOLOGY: linear

(ii) MOLECULE TYPE: N

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Glu Asp His Met Phe Gly Val Gln Gln Ile Gln Pro Asn Val Ile  
 1 5 10 15  
 Ser Val Arg Leu Phe Lys Arg Lys Val Gly Gly Leu Gly Phe Leu Val  
 20 25 30  
 Lys Glu Arg Val Ser Lys Pro Pro Val Ile Ile Ser Asp Leu Ile Arg  
 35 40 45  
 Gly Gly Ala Ala Glu Gln Ser Gly Leu Ile Gln Ala Gly Asp Ile Ile  
 50 55 60  
 Leu Ala Val Asn Gly Arg Pro Leu Val Asp Leu Ser Tyr Asp Ser Ala  
 65 70 75 80  
 Leu Glu Val Leu Arg Gly Ile Ala Ser Glu Thr His Val Val Leu Ile  
 85 90 95  
 Leu Arg Gly Pro Glu Gly Phe Thr Thr His Leu Glu Thr Thr Phe Thr  
 100 105 110  
 Gly Asp Gly Thr Pro Lys Thr Ile Arg Val Thr Gln Pro Leu Gly Pro  
 115 120 125  
 Pro Thr Lys Ala Val Asp Leu Ser His Gln Pro Pro Ala Gly Lys Glu  
 130 135 140  
 Gln Pro Leu Ala Val Asp Gly Ala Ser Gly Pro Gly Asn Gly Pro Gln  
 145 150 155 160  
 His Ala Tyr Asp Asp Gly Gln Glu Ala Gly Ser Leu Pro His Ala Asn  
 165 170 175  
 Gly Leu Ala Pro Arg Pro Pro Gly Gln Asp Pro Ala Lys Lys Ala Thr  
 180 185 190  
 Arg Val Ser Leu Gln Gly Arg Gly Glu Asn Asn Glu Leu Leu Lys Glu  
 195 200 205  
 Ile Glu Pro Val Leu Ser Leu Leu Thr Ser Gly Ser Arg Gly Val Lys  
 210 215 220  
 Gly Gly Ala Pro Ala Lys Ala Glu Met Lys Asp Met Gly Ile Gln Val  
 225 230 235 240  
 Asp Arg Asp Leu Asp Gly Lys Ser His Lys Pro Leu Pro Leu Gly Val  
 245 250 255  
 Glu Asn Asp Arg Val Phe Asn Asp Leu Trp Gly Lys Gly Asn Val Pro  
 260 265 270  
 Val Val Leu Asn Asn Pro Tyr Ser Glu Lys Glu Gln Pro Pro Thr Ser  
 275 280 285  
 Gly Lys Gln Ser Pro Thr Lys Asn Gly Ser Pro Ser Lys Cys Pro Arg  
 290 295 300  
 Phe Leu Lys Val Lys Asn Trp Glu Thr Glu Val Val Leu Thr Asp Thr  
 305 310 315 320  
 Leu His Leu Lys Ser Thr Leu Glu Thr Gly Cys Thr Glu Tyr Ile Cys  
 325 330 335  
 Met Gly Ser Ile Met His Pro Ser Gln His Ala Arg Arg Pro Glu Asp  
 340 345 350  
 Val Arg Thr Lys Gly Gln Leu Phe Pro Leu Ala Lys Glu Phe Ile Asp  
 355 360 365  
 Gln Tyr Tyr Ser Ser Ile Lys Arg Phe Gly Ser Lys Ala His Met Glu  
 370 375 380  
 Arg Leu Glu Glu Val Asn Lys Glu Ile Asp Thr Thr Ser Thr Tyr Gln  
 385 390 395 400  
 Leu Lys Asp Thr Glu Leu Ile Tyr Gly Ala Lys His Ala Trp Arg Asn  
 405 410 415  
 Ala Ser Arg Cys Val Gly Arg Ile Gln Trp Ser Lys Leu Gln Val Phe  
 420 425 430

Asp Ala Arg Asp Cys Thr Thr Ala His Gly Met Phe Asn Tyr Ile Cys  
 435 440 445  
 Asn His Val Lys Tyr Ala Thr Asn Lys Gly Asn Leu Arg Ser Ala Ile  
 450 455 460  
 5 Thr Ile Phe Pro Gln Arg Thr Asp Gly Lys His Asp Phe Arg Val Trp  
 465 470 475 480  
 Asn Ser Gln Leu Ile Arg Tyr Ala Gly Tyr Lys Gln Pro Asp Gly Ser  
 485 490 495  
 10 Thr Leu Gly Asp Pro Ala Asn Val Gln Phe Thr Glu Ile Cys Ile Gln  
 500 505 510  
 Gln Gly Trp Lys Pro Pro Arg Gly Arg Phe Asp Val Leu Pro Leu Leu  
 515 520 525  
 Leu Gln Ala Asn Gly Asn Asp Pro Glu Leu Phe Gln Ile Pro Pro Glu  
 530 535 540  
 15 Leu Val Leu Glu Val Pro Ile Arg His Pro Lys Phe Glu Trp Phe Lys  
 545 550 555 560  
 Asp Leu Gly Leu Lys Trp Tyr Gly Leu Pro Ala Val Ser Asn Met Leu  
 565 570 575  
 20 Leu Glu Ile Gly Gly Leu Glu Phe Ser Ala Cys Pro Phe Ser Gly Trp  
 580 585 590  
 Tyr Met Gly Thr Glu Ile Gly Val Arg Asp Tyr Cys Asp Asn Ser Arg  
 595 600 605  
 Tyr Asn Ile Leu Glu Glu Val Ala Lys Lys Met Asn Leu Asp Met Arg  
 610 615 620  
 25 Lys Thr Ser Ser Leu Trp Lys Asp Gln Ala Leu Val Glu Ile Asn Ile  
 625 630 635 640  
 Ala Val Leu Tyr Ser Phe Gln Ser Asp Lys Val Thr Ile Val Asp His  
 645 650 655  
 His Ser Ala Thr Glu Ser Phe Ile Lys His Met Glu Asn Glu Tyr Arg  
 660 665 670  
 30 Cys Arg Gly Gly Cys Pro Ala Asp Trp Val Trp Ile Val Pro Pro Met  
 675 680 685  
 Ser Gly Ser Ile Thr Pro Val Phe His Gln Glu Met Leu Asn Tyr Arg  
 690 695 700  
 35 Leu Thr Pro Ser Phe Glu Tyr Gln Pro Asp Pro Trp Asn Thr His Val  
 705 710 715 720  
 Trp Lys Gly Thr Asn Gly Thr Pro Thr Lys Arg Arg Ala Ile Gly Phe  
 725 730 735  
 40 Lys Lys Leu Ala Glu Ala Val Lys Phe Ser Ala Lys Leu Met Gly Gln  
 740 745 750  
 Ala Met Ala Lys Arg Val Lys Ala Thr Ile Leu Tyr Ala Thr Glu Thr  
 755 760 765  
 Gly Lys Ser Gln Ala Tyr Ala Lys Thr Leu Cys Glu Ile Phe Lys His  
 770 775 780  
 45 Ala Phe Asp Ala Lys Val Met Ser Met Glu Glu Tyr Asp Ile Val His  
 785 790 795 800  
 Leu Glu His Glu Thr Leu Val Leu Val Val Thr Ser Thr Phe Gly Asn  
 805 810 815  
 50 Gly Asp Pro Pro Glu Asn Gly Glu Lys Phe Gly Cys Ala Leu Met Glu  
 820 825 830  
 Met Arg His Pro Asn Ser Val Gln Glu Glu Arg Lys Ser Tyr Lys Val  
 835 840 845  
 Arg Phe Asn Ser Val Ser Ser Tyr Ser Asp Ser Gln Lys Ser Ser Gly  
 850 855 860

Asp Gly Pro Asp Leu Arg Asp Asn Phe Glu Ser Ala Gly Pro Leu Ala  
 865 870 875 880  
 Asn Val Arg Phe Ser Val Phe Gly Leu Gly Ser Arg Ala Tyr Pro His  
 885 890 895  
 Phe Cys Ala Phe Gly His Ala Val Asp Thr Leu Leu Glu Glu Leu Gly  
 5 900 905 910  
 Gly Glu Arg Ile Leu Lys Met Arg Glu Gly Asp Glu Leu Cys Gly Gln  
 915 920 925  
 Glu Glu Ala Phe Arg Thr Trp Ala Lys Lys Val Phe Lys Ala Ala Cys  
 930 935 940  
 10 Asp Val Phe Cys Val Gly Asp Asp Val Asn Ile Glu Lys Ala Asn Asn  
 945 950 955 960  
 Ser Leu Ile Ser Asn Asp Arg Ser Trp Lys Arg Asn Lys Phe Arg Leu  
 965 970 975  
 15 Thr Phe Val Ala Glu Ala Pro Glu Leu Thr Gln Gly Leu Ser Asn Val  
 980 985 990  
 His Lys Lys Arg Val Ser Ala Ala Arg Leu Leu Ser Arg Gln Asn Leu  
 995 1000 1005  
 Gln Ser Pro Lys Ser Ser Arg Ser Thr Ile Phe Val Arg Leu His Thr  
 1010 1015 1020  
 20 Asn Gly Ser Gln Glu Leu Gln Tyr Gln Pro Gly Asp His Leu Gly Val  
 1025 1030 1035 1040  
 Phe Pro Gly Asn His Glu Asp Leu Val Asn Ala Leu Ile Glu Arg Leu  
 1045 1050 1055  
 25 Glu Asp Ala Pro Pro Val Asn Gln Met Val Lys Val Glu Leu Leu Glu  
 1060 1065 1070  
 Glu Arg Asn Thr Ala Leu Gly Val Ile Ser Asn Trp Thr Asp Glu Leu  
 1075 1080 1085  
 Arg Leu Pro Pro Cys Thr Ile Phe Gln Ala Phe Lys Tyr Tyr Leu Asp  
 1090 1095 1100  
 30 Ile Thr Thr Pro Pro Thr Pro Leu Gln Leu Gln Gln Phe Ala Ser Leu  
 1095 1110 1115 1120  
 Ala Thr Ser Glu Lys Glu Lys Gln Arg Leu Leu Val Leu Ser Lys Gly  
 1125 1130 1135  
 Leu Gln Glu Tyr Glu Glu Trp Lys Trp Gly Lys Asn Pro Thr Ile Val  
 35 1140 1145 1150  
 Glu Val Leu Glu Glu Phe Pro Ser Ile Gln Met Pro Ala Thr Leu Leu  
 1155 1160 1165  
 Leu Thr Gln Leu Ser Leu Leu Gln Pro Arg Tyr Tyr Ser Ile Ser Ser  
 1170 1175 1180  
 40 Ser Pro Asp Met Tyr Pro Asp Glu Val His Leu Thr Val Ala Ile Val  
 1185 1190 1195 1200  
 Ser Tyr Arg Thr Arg Asp Gly Glu Gly Pro Ile His His Gly Val Cys  
 1205 1210 1215  
 45 Ser Ser Trp Leu Asn Arg Ile Gln Ala Asp Glu Leu Val Pro Cys Phe  
 1220 1225 1230  
 Val Arg Gly Ala Pro Ser Phe His Leu Pro Arg Asn Pro Gln Val Pro  
 1235 1240 1245  
 Cys Ile Leu Val Gly Pro Gly Thr Gly Ile Ala Pro Phe Arg Ser Phe  
 1250 1255 1260  
 50 Trp Gln Gln Arg Gln Phe Asp Ile Gln His Lys Gly Met Asn Pro Cys  
 1265 1270 1275 1280  
 Pro Met Val Leu Val Phe Gly Cys Arg Gln Ser Lys Ile Asp His Ile  
 1285 1290 1295

Tyr Arg Glu Glu Thr Leu Gln Ala Lys Asn Lys Gly Val Phe Arg Glu  
 1300 1305 1310  
 Leu Tyr Thr Ala Tyr Ser Arg Glu Pro Asp Lys Pro Lys Lys Tyr Val  
 1315 1320 1325  
 5 Gln Asp Ile Leu Gln Glu Gln Leu Ala Glu Ser Val Tyr Arg Ala Leu  
 1330 1335 1340  
 Lys Glu Gln Gly Gly His Ile Tyr Val Cys Gly Asp Val Thr Met Ala  
 345 1350 1355 1360  
 Ala Asp Val Leu Lys Ala Ile Gln Arg Ile Met Thr Gln Gln Gly Lys  
 1365 1370 1375  
 10 Leu Ser Ala Glu Asp Ala Gly Val Phe Ile Ser Arg Met Arg Asp Asp  
 1380 1385 1390  
 Asn Arg Tyr His Glu Asp Ile Phe Gly Val Thr Leu Arg Thr Tyr Glu  
 1395 1400 1405  
 15 Val Thr Asn Arg Leu Arg Ser Glu Ser Ile Ala Phe Ile Glu Glu Ser  
 1410 1415 1420  
 Lys Lys Asp Thr Asp Glu Gly Phe Gln Leu Leu Thr Gly Pro Ser Cys  
 425 1430 1435 1440  
 20 Pro Ala Gly Cys Lys Phe Cys Lys Arg Gly Gln Thr Leu Leu Asn Leu  
 1445 1450 1455  
 Ser Ser Gly Thr Pro Cys Gly Pro Arg Ser Ala Ser Cys Pro Cys Arg  
 1460 1465 1470  
 Cys Ala Leu Val Ser Leu Leu Gly Leu Leu Ala Pro Gln Trp Phe Pro  
 1475 1480 1485  
 25 Arg Pro Ser Trp Val Tyr Ser Leu Ser Phe Pro Ala Ala Met Gln Cys  
 1490 1495 1500  
 Phe Ser Asn Leu Gln Trp Leu Leu Gln Asn Ser Val Pro Thr Pro Ser  
 505 1510 1515 1520  
 30 Leu Ala Asp Lys Gly Asn Ser Arg Val His Glu Thr Thr Gly Thr Trp  
 1525 1530 1535  
 Pro Ser Leu Trp Gly Phe Phe Ser Leu Gly Phe Pro Trp Lys Gly Cys  
 1540 1545 1550  
 Arg Asn

35 (2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs  
 (B) TYPE: nucleic acid  
 40 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

30 GACTAGTCGA CTGAAGAGAA CACGTTGGG

45 (2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs  
 (B) TYPE: nucleic acid

WO 99/37768

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

5 TCTGCAGATC TCAGTGGGCC TTGGAGCCAA A  
31

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/01199

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/12 C07K14/47 G01N33/68 C12Q1/68 C12N15/62  
C07K16/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07K C12N G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JAFFREY S R ET AL: "PIN: AN ASSOCIATED PROTEIN INHIBITOR OF NSURONAL NITRIC OXIDE SYNTHASE" SCIENCE, vol. 274, 1 November 1996, pages 774-777, XP002050141 cited in the application see the whole document ---	
A	BRENMAN J E ET AL.: "Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alphasyntrophin mediated by PDZ domains" CELL, vol. 84, 8 March 1996, pages 757-767, XP002104701 cited in the application see the whole document ---	

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Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

3 June 1999

Date of mailing of the international search report

16/06/1999

Name and mailing address of the ISA

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NL - 2280 HV Rijswijk  
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Fax: (+31-70) 340-3016

Authorized officer

Oderwald, H

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/01199

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>JAFFREY S R ET AL.: "CAPON: a protein associated with neuronal nitric oxide synthase that regulates its interaction with PSD95"          NEURON,          vol. 20, no. 1, 23 January 1998, pages          115-124, XP002104610          see the whole document          -&amp; "accession number AF037070"          EMBL SEQUENCE DATABASE, 20 February 1998,          XP002104702          Heidelberg, Germany          see the whole document          -&amp; "accession number AF037071"          EMBL SEQUENCE DATABASE, 20 February 1998,          XP002104703          Heidelberg, Germany          see the whole document</p> <p>-----</p>	1-39

## INTERNATIONAL SEARCH REPORT

Int. application No.

PCT/US 99/01199

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:

because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 25-27

are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2.  Claims Nos.:

because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3.  Claims Nos.:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

1920-1921

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1920-1921